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The Diagnosis, Genetics and Management of Patients with Cystinuria

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The Diagnosis, Genetics and Management of Patients with Cystinuria

A thesis submitted to Kings College
London
for the degree of MD (Res)

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December 2017

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NHS Foundation Trust

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I would like to thank Matthew Bultitude who has helped to proofread the key papers in my thesis. I would like to thank all my research collaborators who I have mentioned in the relevant chapters of my thesis for their contributions. In particular, I would like to thank Mark Wass and Rachael Mein, both of whom have been instrumental in my understanding of genetics and protein modelling.

I would also like to thank the specialist Cystinuria team including Angela Doherty, Giles Rottenberg, Mitra Smith, and Morloh Kabia. I would like to thank all the patients who have participated in this research and helped to further my understanding of their disease.

Last but not least, I would not be where I am without the daily support and encouragement from my husband Christopher Rao.

Declaration of Originality

The work presented in this thesis is my own work, supported by my supervisors. Where thoughts, ideas and work of others are presented, every effort has been made to ensure that it is appropriately acknowledged and reference.

Contributions

Dr Erin Mozley conducted a small study assessing different methods of cystine quantification. Dr Charles Turner helped me in preparation of the samples for the experiments.

Dr Caroline Pardy contributed data collection and analysis to chapter 3.

Dr Soma Pillay, Dr Ashish Chandra and their team are responsible for the cytology reporting and contributed to chapter 4.

Dr Rachael Mein performed the DNA sequencing and MLPA work and contributed to chapter 5 and 6.

Dr Mark Wass performed the protein modelling in chapter 6.

Abstract

Cystinuria is a genetic disease leading a defective dibasic amino acid transporter in the renal proximal tubules resulting in an accumulation of urinary cystine. Urinary cystine precipitates into crystals which is believed to be a necessary step to stone formation. There is a wide variation in disease presentation that is not well understood and cannot be explained by either compliance with medical and dietary interventions, or differences in patient management. Predicting disease severity and managing patients expectantly is confounded by a paucity of validated methods to monitor disease activity and heterogeneity in how urinary dibasic amino acid levels are measured and reported in literature.

Management of this disease is largely preventative and based on historical data. At Guy's and St Thomas', a clinic was set up to allow for a multidisciplinary approach to the management of these patients. It also provided a premise for research into patients with this disease.

The objective of my research was threefold; to understand the factors that lead to severe disease, to investigate clinical markers of disease activity, and to understand the genetic mutations that cause the disease.

This thesis is divided into seven chapters incorporating five supporting publications. Chapter one details what is already known on the subject. The evidence for dietary recommendations, current medical therapy and treatments are discussed. Chapter two describes the challenges to the clinician in the management of this disease. The basis of the specialist multidisciplinary clinic is

outlined, the roles of each team member and the geographic distribution of our patient cohort.

Chapter three investigates the utility of dibasic amino acids in the management of cystinuria in particular, the association between urinary dibasic amino acids levels and stone formation. Dr Caroline Pardy and Dr Erin Mozley contributed significantly to this chapter. Spot urine samples were collected at the time of each clinic visit. The levels of the urinary dibasic amino acids and the association with stone formation as evaluated by ultrasound were analysed. There was a statistically significant association between the levels of urinary ornithine and the presence of stones seen on ultrasound scan. However, as current cystinuria medications aim at reducing cystine levels only with no known effects on levels of urinary ornithine, the prognostic value of this remains uncertain.

Chapter four describes original research on the diagnostic value of crystalluria. Separate early morning and clinic urine were collected at each clinic visit and the association between presence of crystals and presence of stones and new stone growth were analysed. Malassez counting chamber and conventional cytopsin methods were also compared. The results demonstrated that the presence of crystals in patients with cystinuria is associated with stone formation and new stone growth when based on clinic urine using cytopsin method. This may serve as a useful adjunct rather than as a single diagnostic tool.

For the first time, the genetic mutations found in a UK population are characterized and described in chapter five. Dr Rachael Mein contributed

significantly to this work. We found 23 new mutations in our UK population. We have found that in patients with mutations in SLC3A1, the presence of a missense mutation leads to lower levels of urinary lysine, ornithine and arginine. This is the first time such a genotype-phenotype association has been found and has the potential to improve our risk stratification of patients at the time of diagnosis and tailor their subsequent follow up. This association was not seen for cystine levels however there are limitations in current cystine assays that may account for this and is further discussed in the chapter.

In chapter six, the use of protein modelling to model the two proteins encoded by SLC3A1 and SLC7A9 and the mutations that lead to protein dysfunction is described. The severity of the mutations in SLC7A9 as determined by the proximity of the mutation to the ligand binding sites and size of conformational change is shown to lead to a more severe biochemical phenotype as evidenced by raised levels of urinary dibasic amino acids. Further work in this area has the potential to lead to a personalized approach to the management of this disease.

Finally, chapter seven summarises all the research findings, discusses the implications to current practice and future challenges in the management of these patients.

Contribution to the Science of Urinary Calculus Disease

Original Research Supporting Thesis

Wong KA, Pardy C, Pillay S, Athanasiou T, Rottenberg G, Bultitude M, Chandra A, Thomas K. *Can the presence of crystalluria predict stone formation in patients in Cystinuria?* Journal of Endourology 2016 Jan 18. PMID 26781171

Wong, KA, Wass M, Thomas K. *The Role of Protein Modelling in Predicting the Disease Severity of Cystinuria.* Eur Urol. 2016 Mar;69(3):543-4. doi: 10.1016/j.eururo.2015.10.039. Epub 2015 Nov 14. PMID 26589650

Wong KA, Mein R, Wass M, Flinter F, Pardy C, Bultitude M, Thomas K. *The genetic diversity of cystinuria in a UK population of patients.* BJU Int 2015, 116(1):109-16. PMID 25109415

Wong KA, Sahai A, Patel A, Thomas K, Bultitude M, Glass J. *Is Percutaneous Nephrolithotomy in solitary kidneys safe?* Urology 2013 Nov;82(5):1013-6 PMID 23958507

Published Review Article

Thomas K, **Wong KA**, Withington J, Bultitude M, Doherty A. *Cystinuria – A Urologist’s Perspective*. Nat Rev Urol 2014, 11(5):270-277. PMID 24662732

Presentations and Published Abstracts

Kum F, **Wong KA**, Game D, Glass J, Bultitude M, Thomas K. *Hypertension and renal impairment in patients with cystinuria: Findings from a specialist cystinuria centre*. Poster presentation. European Urology Supplements 15(3)e911 Mar 2016

Wong KA, Wass M, Mein R, Flinter F, Bultitude M, Thomas K. *A personalized approach to the management of Cystinuria*. Invited Presentation. The Royal Society of Medicine Innovation Day Feb 2016.

Wong KA, Wass M, Mein R, Flinter F, Bultitude M, Thomas K.
Understanding the Genes. Invited Presentation. National Cystinuria
Patient Day April 2015.

Patel S, **Wong KA**, Rottenberg G, Mein R, Bultitude M, Thomas K.
Evaluation of Hounsfield Units in Cystinuria patients. Poster
presentation. BJU International Vol 113 June 2014

Doherty A, **Wong KA**, Kamoga G, Bultitude M, Pardy C, Thomas K.
*Patient Satisfaction and Dietary Outcomes from Attending a
Multidisciplinary Cystinuria Clinic*. Poster presentation. The
International Congress in Nutrition and Metabolism in Renal Disease
(Abstract) 2012.

Wong KA, Mein R, Wass M, Pardy C, Kabia M, Smith M, Flinter F,
Thomas K. *The Detection of New Pathogenic Mutations in
Cystinuria*. Podium presentation. British Association of Urological
Surgeons Annual Meeting Glasgow Jun 2012. **Best of BAUS prize**

Wong KA, Mein R, Wass M, Pardy C, Kabia M, Smith M, Flinter F,
Thomas K. *The Detection of New Pathogenic Mutations in*

Cystinuria. Podium presentation. Urology Short Papers section at the Royal Society of Medicine Mar 2012. **First Prize**

Wong KA, Mein R, Wass M, Pardy C, Kabia M, Smith M, Flinter F, Thomas K. *The Detection of New Pathogenic Mutations in Cystinuria*. Podium Presentation. American Urological Association May 2012

Wong KA, Mein R, Wass M, Pardy C, Kabia M, Smith M, Flinter F, Thomas K. *The Detection of New Pathogenic Mutations in Cystinuria*. Poster Presentation. European Association of Urology Paris Feb 2012

Wong KA, Pardy C, Pillay S, Kabia M, Chandra A, Thomas K. *Evaluation of Cystine Crystalluria in the First Void versus Clinic Urine Specimen in Cystinurics using Different Counting Methods*. Podium Presentation. South Thames Regional Meeting Nov 2011.

Pardy C, **Wong KA**, Doherty A, Kabia M, Bultitude M, Rottenberg G, Moxham V, Thomas K. *Urinary ornithine, arginine, and lysine may be better predictors of cystine stone formation and progression than urinary cystine*. J Urol 185(4) April 2011

Pardy C, **Wong KA**, Doherty A, Kabia M, Bultitude M, Rottenberg G, Moxham V, Thomas K. *Urinary lysine is a better predictor of cystine stone formation and progression than urinary cystine.*
European Urology Supplements 10(2):227-227 Mar 2011

Prizes

First Author

- **Best of BAUS award** *The Detection of New Pathogenic Mutations in Cystinuria*. Podium presentation. British Association of Urological Surgeons Annual Meeting Glasgow Jun 2012
- **First Prize** *The Detection of New Pathogenic Mutations in Cystinuria*. Malcolm Copcoat Urology Short Papers section. Royal Society of Medicine Mar 2012

Second Author

- **Best of AUA** *Urinary ornithine, arginine and lysine may be better predictors of cystine stone formation and progression than urinary cystine*. American Urological Association 2011
- **Best Poster** *Urinary lysine is a better predictor of cystine stone formation and progression than urinary cystine*. European Association of Urology 2011
- **First Prize** *Urinary lysine, arginine and ornithine, may be better predictors of cystine stone formation than urinary*

cystine and urinary cystine crystals. Royal Society of
Medicine Urology Clinicopathology Meeting 2011

Abbreviations

B(0+)AT - Light Chain b Amino Acid Transporter

CDME - L-cystine dimethylester

CU – Clinic Urine

DNA – Deoxyribonucleic Acid

EMU – Early Morning Urine

ESWL – Extracorporeal Shockwave Lithotripsy

HPLC - High Performance Liquid Chromatography (HPLC)

LC-MS - Liquid Chromatography-tandem Mass Spectrometry

MLPA – Multiplex Ligand-dependent Probe Amplification

NPV – Negative Predictive Value

NSF – New Stone Former

NSG – New Stone Growth

PCNL – Percutaneous Nephrolithotomy

PCR – Polymerase Chain Reaction

RaDaR – National Registry of Rare Kidney Disease

rBAT - Neutral and Basic Amino Acid Transport Protein

ROC – Receiver Operating Characteristic

URS – Ureteroscopy

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1 Introduction

1.1 Cystinuria is a genetic disease

Cystinuria is an inherited dibasic aminoaciduria. A mutation in one of two genes SLC3A1 and/or SLC7A9 results in defective transport of urinary cystine and the dibasic amino acids across the small intestine and renal tubular membrane. In the kidneys, this results in an accumulation of urinary cystine, lysine, arginine and ornithine. As cystine is relatively insoluble compared to the other dibasic amino acids, high levels in urine can precipitate to form crystals and eventually stones and lead to stone-related complications³.

The clinical presentation is varied; ranging from some patients having stone episodes every few months to other patients having only one stone in their lifetime. A large stone burden may result in chronic kidney disease and even nephrectomy⁵⁻⁷. Cystinuria represents 1% of adult and 6–8% of paediatric stone disease, although the prevalence varies between populations⁸. The prevalence of cystinuria is likely to be underestimated in many studies because patients with mild disease may never present with stone disease or are not screened for cystinuria.

The recurrence rate of stone episodes is reported to be 60%.⁹ Men have been reported to have more severe disease than women and form stones more frequently.¹⁰ Reports in the literature suggest that three-quarters of patients with cystinuria develop bilateral stones;¹¹ however, in our own cohort, we have found only one third of patients with bilateral disease.



Figure 1.1 The first cystine stone as described by Wollaston in 1810. (Courtesy of the Gordon Museum, Guys Campus Kings College London)

| | |
|---|-----------|
| Total Number of patients | 86 |
| | |
| Male: Female | 1.32 |
| Average age of presentation | 23 |
| Number of patients with a delayed diagnosis of Cystinuria | 23 |
| Average delay in diagnosis (years) | 7.8 |
| Passes stones spontaneously | 46 |
| Bilateral stones | 26 |
| Radio opaque stones | 16/30 |
| Respond to ESWL | 15/32 |
| Hypertensive | 28 |
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Table 1.1 Patient Demographics from the Guys and St Thomas' Cystinuria Clinic. Out of 30 patients who have undergone X-ray imaging, just over half (N=16) have stones that are radio-opaque. Therefore, we use ultrasound as our preferred mode of imaging to detect cystine stones. Furthermore, only 15 out of 32 patients respond to ESWL treatment of their stones. We routinely offer ESWL treatment to these patients who have been known to respond in the past. Patients who fail ESWL treatment are generally offered more invasive treatment i.e. URS/PCNL.

1.1.1 Diagnosis

The initial diagnosis of cystinuria is made by stone analysis showing cystine composition and raised levels of urinary dibasic amino acids. Commonly, sodium cyanide-nitroprusside is used as a screening test and only requires a urine sample. Cyanide reduces cystine to cysteine which then binds to nitroprusside resulting in a red to purple colour change¹². The sensitivity of the test has been reported to be 72% with a specificity of 95%.¹²

As it is a rare disease, patients often present several times with stone disease before a diagnosis is made. In our experience, from our own cohort of patients who have been referred to our specialist clinic, the average delay in diagnosis was 7.8 years³.

Recent evidence suggests that hypertension occurs more frequently in patients with cystinuria than the general population and therefore these patients should be routinely screened⁶. We have also found in our patients 28/86 are hypertensive and have diagnosed a further 26% (22/86) patients with hypertension requiring treatment³.

1.1.2 Genetics

Mutations in SLC3A1 and/or SLC7A9 explain the majority of cases of Cystinuria. SLC3A1 encodes for the neutral and basic amino acid transport protein (rBAT) and SLC7A9 encodes for the light chain b amino acid transporter b(0+)-AT^{13,14}. The proteins form a dimer linked by a disulphide bridge¹⁵. b(0+)-AT consists of 487 amino acids (53481 Da) with 12 transmembrane helices

that form the channel through which dibasic amino acids (cystine, lysine, arginine and ornithine) are transported into the cell with the exchange of neutral amino acids. rBAT is the larger of the two subunits consisting of 685 amino acids (78852 Da), with a single transmembrane helix and a large extracellular domain. Experimental studies suggest that rBAT may function as an activator of b(0+)_{AT}^{14,16}.

SLC3A1 mutations are inherited in an autosomal recessive pattern whilst mutations in SLC7A9 can be regarded as inherited in an autosomal dominant pattern with incomplete penetrance¹⁷. In SLC3A1, mutations in both alleles of the gene are required for disease presentation. In SLC7A9, mutations in both alleles can result in cystinuria however patients with only one mutated allele can also form cystine stones¹⁰.

1.2 Current Medical Therapy and Treatments

1.2.1 Preventing stone formation

Preventing stone formation is an important focus for clinicians undertaking specialist long-term cystinuria management and care. The urologist's aim is to minimize the frequency of renal colic episodes, the need for urological interventions and the adverse sequelae of recurrent stone formation.

The ratio of soluble to insoluble cystine is affected by its urinary concentration, urinary pH, and urine volume¹⁸. Cystinuria is more likely to lead to long-term renal damage than other stone diseases^{19,20} and, therefore, our clinic has employed a multidisciplinary approach to managing these patients.

1.2.2 Hydration

Adequate hydration is the simplest and most important method to prevent stone formation in cystinuria. Reducing cystine concentration in urine can be achieved by hyperdiuresis. If patients with cystinuria excrete up to 1400 mg of cystine per day and the solubility of cystine is approximately 300 mg/l at pH 7.0, then a urine output of 5 l per day should enable the total clearance of cystine.²¹ However, this level of fluid intake is not sustainable for most patients and a target of exceeding 3 l per day is more realistic^{22,23}. Specific regimens might also be recommended, often involving waking at night to drink a specified volume of fluid.^{21,22,24} Traditionally, night was thought to be when most patients were at highest risk of cystine supersaturation.²⁵ However, studies have not shown a diurnal difference in the number of discrete supersaturation episodes.²⁶

We ask our patients to complete a diary describing fluid intake and our specialist dietician provides tailored advice regarding fluid management depending on patient's individual circumstances.²⁴ Other researchers have found that patients struggle to maintain a high fluid intake, but improved with frequent monitoring and feedback during 6-monthly clinics.²⁷ In our own experience, the frequency of follow up is varied depending on patient need and response. 58% of our patients have increased their fluid intake since attending our clinic; 66% of our patients are maintaining a fluid intake of greater than 3 l per day and 13% 4 l and above¹.

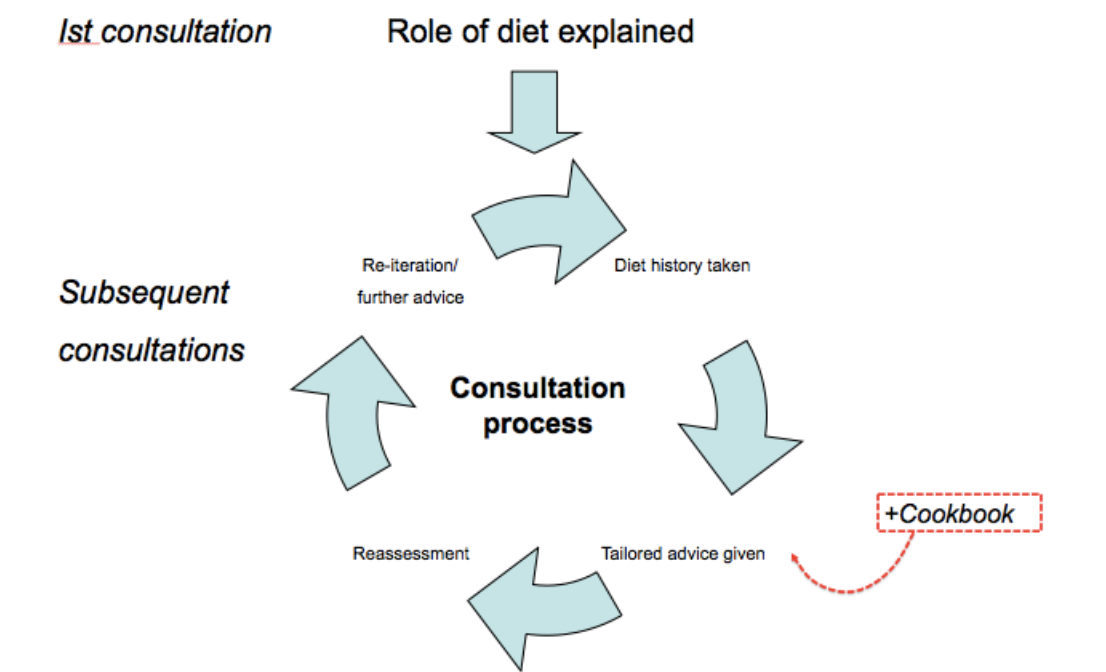


Figure 1.3 Diagram of the Guys diet clinic model (adapted from Wong et al 2012¹)

Alkalizing beverages, such as mineral water, which is rich in bicarbonate and low in sodium (1500 mg bicarbonate per l, maximum 500 mg sodium per l) and citrus juice are the preferred fluid sources.²¹ There is also evidence suggesting that orange juice is more effective than lemonade in alkalinising urine and promoting hypercitraturia²⁸. The authors of this paper concluded this may be protective in recurrent calcium oxalate and uric stone formers and could be encouraged particularly for patients who are intolerant to potassium citrate. Potentially, the alkalinising effect of orange juice may also be beneficial in cystinuria patients although this has not been tested. To maintain high fluid intake, patients should be advised to drink what they like, provided it mostly consists of a clear fluid, and to avoid caffeinated drinks.²¹

1.2.3 Diet

The recommended diet for cystinuria patients includes maintaining a healthy weight, limiting intake of salt and animal protein, and increasing vegetable protein (as a substitute for animal protein), fruit and vegetables^{22,23,29-35}.

Historically, the ability of patients to adhere to and maintain specific dietary regimens has been questioned²³. However, in our multidisciplinary practice, with regular dietetic input and support, patients have made positive changes to their diets.

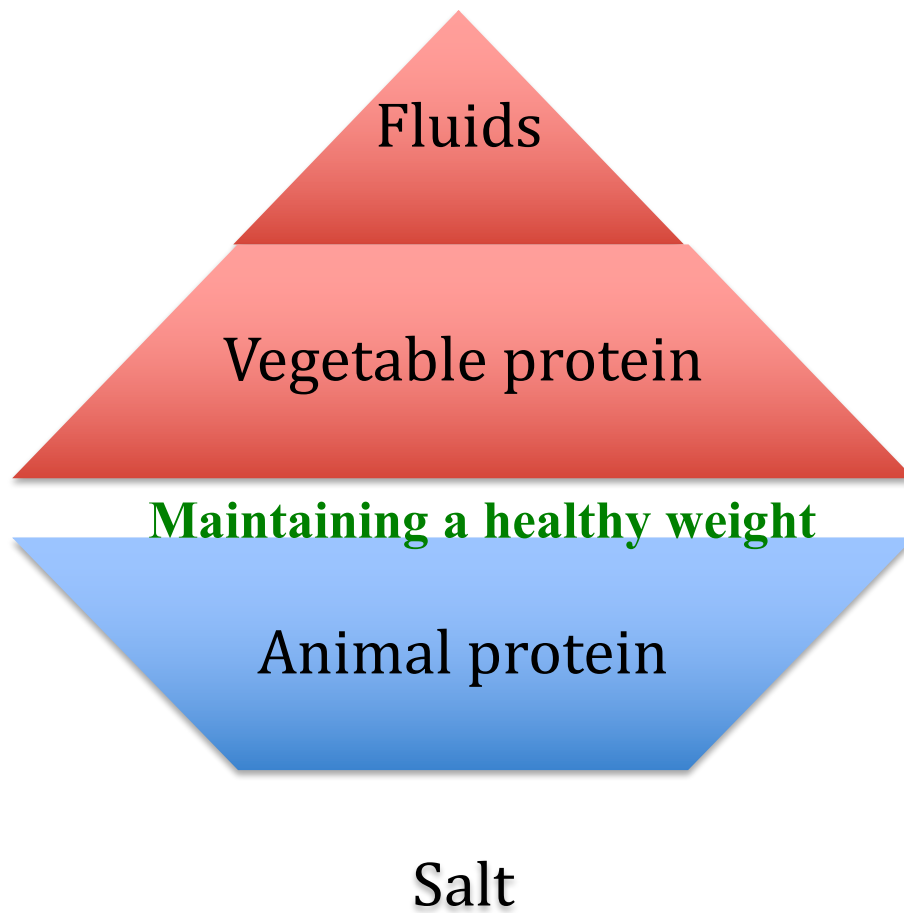


Figure 1.4 Recommended dietary changes for a patient with cystinuria

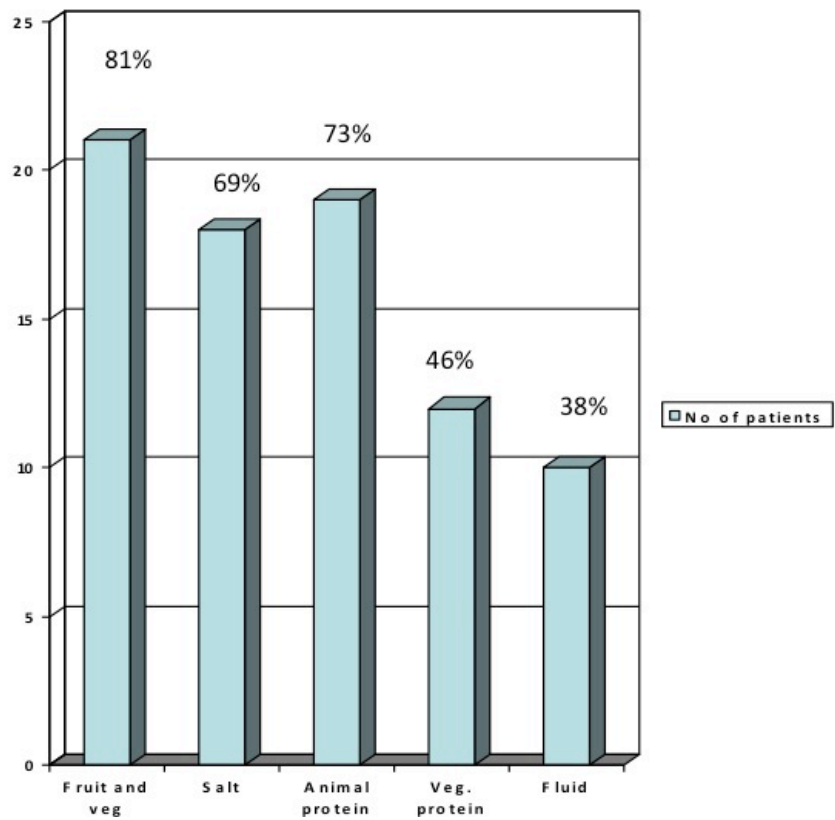


Figure 1.5 Number of patients making positive changes to their diet after review (Adapted from Wong et al 2012¹)

Urinary pH is also inversely related to body weight,³⁶ overweight patients, therefore, have a less favourable urine pH, which is conducive to stone formation. 30% of patients in our cohort, when first seen in clinic, were overweight (defined as a BMI 25.1–30 kg/m²) and 28% were clinically obese (BMI >30kg/m²). With the regular dietetic support, 12.5% of patients in the overweight group and 34.8% of patients in the obese group have lost weight.

Decreased total sodium intake can reduce the amount of cystine excreted in the urine.^{25,31,37} Lowering sodium intake by 3.5 g per day decreases cystine excretion by 156 mg per day, and encourages cystine solubility.³²

In the UK, the average salt intake per day is 8.6 g (3.4 g sodium), 75% of this salt intake comes from processed foods. Patients are encouraged to restrict their salt intake to ≤6 g per day (2.5g of sodium)^{38,39}. Patients are advised to eat more fresh foods, to check food labels in order to choose the lower salt options and stop adding salt to their food, instead experimenting with alternative seasonings. 68% of patients have reduced their salt intake since attending our clinic.

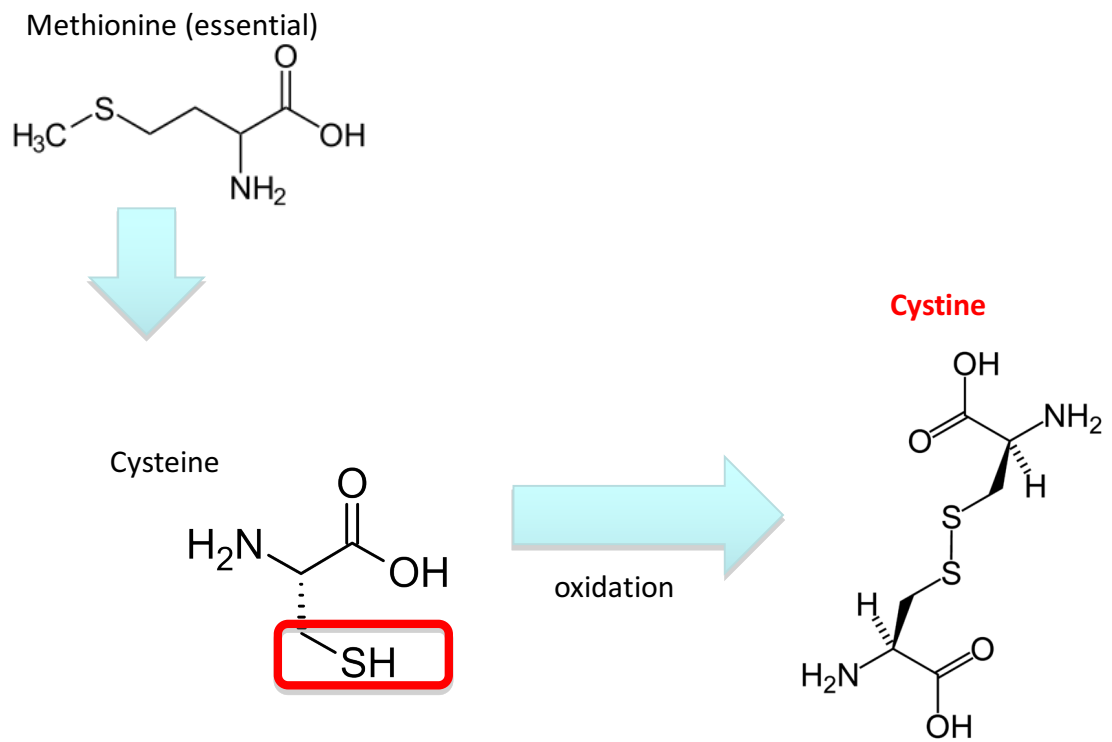


Figure 1.6 Methionine, an essential amino acid found in many food groups such as egg, nuts, fish and meat is a cystine precursor (adapted from Wikipedia.org)

Reducing animal protein intake also decreases cystine excretion by reducing methionine and cystine intake.³³ A reduced methionine—a cystine precursor—diet has been recommended for patients with cystinuria. In one case study, a patient who followed a strict low methionine diet for 10 years found that his cystinuria effectively disappeared.⁴⁰ Moreover, an extremely low protein diet (20 g per day) was found to reduce urinary cystine excretion by 34%.^{22,41} However, this level of protein restriction is not advisable, and may even be harmful to patients^{42,43}.

In our clinic, a stones diet-based guideline is used to help patients achieve a restricted animal protein intake.⁴⁴ Patients are advised to limit their intake of protein from animal sources to 60% of their total protein requirements, the remaining 40% should come from vegetable protein foods and other sources (for example, the small amounts of protein in rice or bread). A patient's daily protein intake is estimated from their diet history and compared with their protein requirements calculated using the Elia equation ($6.25(0.17\text{g/kg body weight/day, adjusted for obese patients with BMI } >30)$)⁴⁵. A cookbook with vegetable protein-based recipes and specific advice for patients with cystinuria was produced by our team.²

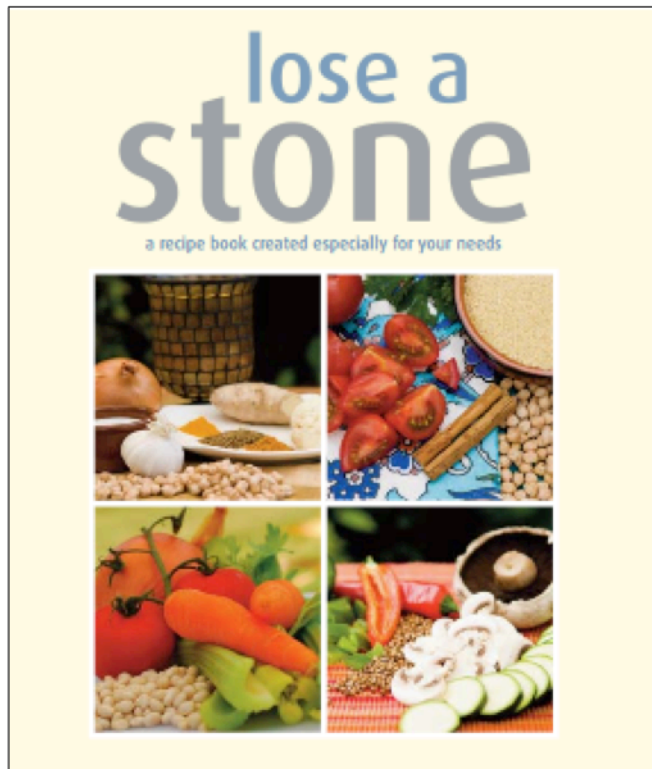


Figure 1.7 Thomas et al. Lose a Stone Cystinuria cookbook²

Following dietary advice, 68% of our patients decreased their animal protein intake and 62% increased their vegetable protein intake. We carefully monitored those patients who decreased their animal protein intake, and without a concurrent increase in vegetable protein, to ensure they consumed enough protein. To determine if a patient was malnourished, we assess grip strength using a dynamometer—a reduction in grip strength indicates protein malnutrition. Decreasing animal protein intake also increases urinary pH, which will help limit cystinuria²⁹, as can increased fruit and vegetable intake owing to the high levels of potential basic amino acids in fruits and vegetables.³⁴ In our clinic, 72% of patients increased their fruit and vegetable intake following dietary advice.

Patients with cystinuria are at risk of forming calcium phosphate stones,⁴⁶ and low calcium diets are known to increase the risk of forming stones.⁴⁷ To ensure that patients with cystinuria receive enough dietary calcium they are advised that at least two sources of protein come from dairy foods.

Dietary changes can reduce levels of cystine in the urine, and some of our patients with cystinuria have had notable success with reducing stone episodes.³³ However, no randomized studies have been conducted to demonstrate that dietary changes reduce stone episodes.

1.2.4 Medical Management

Medical therapy for cystinuria is focused on lowering the urinary concentration of cystine and increasing its solubility.⁴⁸ Aggressive medical management can reduce the incidence of stone formation by 78%.⁴⁸

Cystine precipitates into crystals in acidic urine (pH 5–7)¹⁸ In patients with consistently low urinary pH despite dietary changes and weight loss, an alkalinizing agent can be used. Potassium citrate or sodium bicarbonate can increase urinary pH and the solubility of cystine.⁹ However, in our clinic we prefer to use potassium citrate, which avoids the excess urinary sodium load that arises from bicarbonate therapy and thereby might lead to mixed composite stones. 27 of patients in our cohort receive potassium citrate compared with just 3 on sodium bicarbonate. Patient compliance with potassium citrate is limited due to its poor taste, but can be overcome by using a tablet form (Urocit K©), which unfortunately in the UK is more expensive and difficult to obtain. All patients receiving potassium citrate should have their serum potassium levels and urinary pH checked regularly aiming for a urinary pH of 7–7.5. In our clinic, we provide urine dipsticks and ask all our patients to complete a urinary pH/fluid diary. Excessive alkalinisation of urine to a value consistently >pH 7.5 can predispose patients to calcium phosphate stone formation, which has happened to two of our patients.

In addition to alkalinisation therapy, thiol-binding drugs such as D-penicillamine and α -mercaptopropionylglycine (also known as tiopronin)^{9,23} can be used to treat cystinuria. 30% of our patients are on thiol-binding medications which work

by reducing cystine to two cysteine molecules. A soluble drug-product 50 times more soluble than cystine is formed, which can then be excreted in urine. *In vitro* studies suggest that alkalinisation of urine might also potentiate the effect of these thiol drugs.⁴⁹ Tiopronin is reported to have fewer side effects than D-penicillamine (75.5% vs 83.7%),⁵⁰ leading to improved patient experience and compliance. Side effects include non-specific allergy, nausea, leucopenia, thrombocytopaenia, proteinuria and Systemic Lupus Erythematosus-like syndromes, so patients should be regularly monitored⁴⁸. 9% of our patients have stopped D-penicillamine and 6% tiopronin due to problems of tolerability. Due to the potential side effects, all patients receiving D-penicillamine and tiopronin have 3–6 monthly full blood count, liver function tests, albumin, renal function, vitamin B6 and urine dipstick for protein levels checked. Due to the risk of vitamin B6 deficiency in patients taking D-penicillamine only, vitamin B6 supplements are also recommended.

Captopril is an angiotensin–converting–enzyme inhibitor, more commonly used to control blood pressure and its clinical efficacy in the context of cystinuria is debatable.^{51,52} It works by forming a thiol-cysteine mixed disulphide complex, which is more soluble than cystine. In patients with hypertension, captopril can be used as an alternative or adjunct treatment. In our clinic four patients with cystinuria are on captopril.

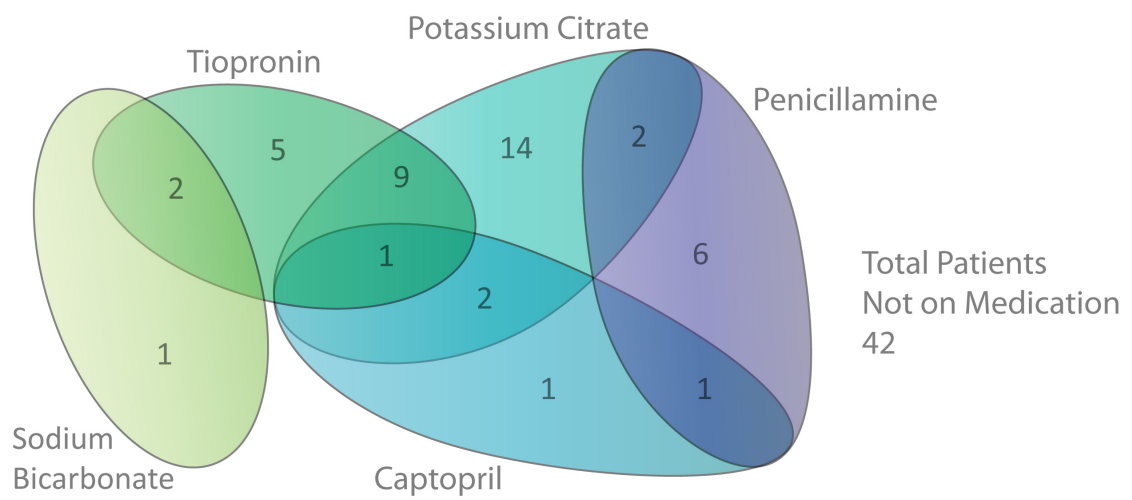


Figure 1.8 The number of patients on drugs in our specialist cystinuria clinic (Thomas, Wong et al 2014³)

1.2.5 Surgical Management

The surgical techniques for removal of cystine stones are identical to all other stone types. The choice of intervention will depend on numerous factors, such as stone size, location, nature of previous interventions, availability of equipment, surgeon and patient preference. Patients with cystinuria have high recurrence rates and therefore surgical planning lies in achieving a balance between the safety of the procedure and stone clearance.²¹

Like all patients who form stones, a substantial proportion of those with cystinuria can spontaneously pass stones before their size becomes problematic. In our cohort, >50% of patients pass stones spontaneously. Those patients who develop recurrent stones will often know from previous experience the maximum size of stone that they are likely pass, smaller asymptomatic stones can, therefore, be monitored. However, prompt treatment is required if stones fail to pass, after developing colic or if the stone grows. Some evidence indicates that complete stone clearance leads to a longer stone-free period.⁵³

1.2.5.1 Extracorporeal shockwave lithotripsy

Extracorporeal shockwave lithotripsy (SWL) is the non-invasive fragmentation of stones into smaller fragments that are more likely to pass. Cystinuria stones are widely believed to be resistant to fragmentation by SWL, which is supported by clinical and *in-vitro* studies, although the results have varied. For example, in one study 8 of 10 patients with cystinuria and ureteric stones were stone free after two sessions of lithotripsy, leading the researchers to recommend that ESWL be considered the first line of treatment for all ureteric stones.⁵⁴ However, other researchers have demonstrated in a paediatric population that stone

fragmentation was inferior in patients with cystinuria and in stones larger than 11mm.⁵⁵ In our own experience, 47% (15/32) of patients have stones that respond to ESWL and we consider it a useful first line treatment for those stones <1cm in patients who have previously had successful ESWL treatment.

In vivo studies have shown that significantly more lithotripsy shockwaves may be required to fragment cystine stones compared with other stone types.⁵⁶ There is also some evidence to suggest that cystine stones with a rough external surface on plain imaging are more amenable to shockwave energy than those with a smooth surface.⁵⁷ Furthermore, rough stones tend to have a lower attenuation coefficient than smooth ones on computerised tomography suggesting that computed tomography might aid the identification of stones suitable for ESWL.⁵⁷

1.2.5.2 Ureterorenoscopy

Rigid ureteroscopy or flexible ureterorenoscopy (also known as retrograde intrarenal surgery) is a suitable treatment modality for the majority of ureteric and pelvicalyceal stones less than 2cm.^{21,58} Ureteroscopic surgery is minimally invasive and confers low morbidity with a short hospital stay, a major advantage and of particular importance for patients with cystinuria, who often undergoing multiple procedures, possibly in the context of having established CKD or a single kidney. The holmium laser can fragment even the hardest stone and many stone surgeons advocate it as the ideal energy source for ureteroscopic surgery⁵⁹.

1.2.5.3 Percutaneous nephrolithotomy

Percutaneous nephrolithotomy (PCNL) might be required for large or complex stones that are not amenable to ESWL or retrograde intrarenal surgery. The threshold for deciding to use PCNL in patients with cystinuria might be higher

than in other stone formers due to the recurrent nature of cystine stone formation and a desire to balance the need for repeated interventions with the risk of complications over a lifetime. PCNL access techniques and sheath size vary but all allow large fragments to be removed and offer a high chance of complete stone clearance²³. Historically, retreatment rates following PCNL are higher for cystinuria stones than for other stone compositions (50% versus 15%).⁶⁰ Indeed, 59% (48/82 out of data available in our cohort) of our patients have previously had a PCNL, therefore, the risks associated with PCNL and the potential renal damage should be considered when planning surgery and the number of PCNL procedures minimised. Our own preference is to use more minimally invasive approaches whenever possible.

A particularly challenging situation arises when patients with cystinuria and a solitary kidney require PCNL. In the CROES study, stone clearance was inferior in patients with single kidneys, who also had poorer post-operative renal function⁶¹. However, in our experience, stone clearance and complication rates were acceptable, with a stone clearance rate of 77% and either an improvement or stabilization in renal function at three months⁶².

1.2.5.4 Percutaneous chemolysis

Historically, some patients with cystinuria have been treated with chemodissolution agents, such as N-acetyl cysteine with bicarbonate, administered by percutaneous irrigation via a nephrostomy tube but with limited success.^{21,63} Concomitant advances in retrograde intrarenal surgery, PCNL and medical prevention have rendered this technique largely obsolete.

1.2.5.5 Open surgery

Advances in minimally invasive stone surgery have almost entirely obviated the need for open surgery in the UK, which is only considered in rare circumstances where minimally invasive approaches have failed or are contraindicated.²¹ Interestingly, 22% of patients in our clinic have had a history of prior open surgery, usually in their childhood or when the disease first presented. This may reflect the number of patients who had a delayed diagnosis at initial presentation and had large volume stone disease. Comparative data on the proportion of cystinuria patients having previously undergone open surgery are not readily available. A history of previous open surgery be reflected in complex anatomy and can make future endourological surgery more challenging, though specific data addressing this issue is lacking.

1.3 Conclusion

Cystinuria is a challenging disease; it affects predominantly a young population and the diagnosis is often delayed as patients present with stone-related complications several times before the possibility of cystinuria is even considered. These patients are more susceptible to stone-related morbidity and chronic renal impairment.

The aims of management of the disease are threefold; firstly to lower urinary cystine levels and increase its solubility through dietary measures and medical therapy. Secondly, to reduced the morbidity associated with stone disease. Finally to minimize irreversible renal dysfunction caused by nephron loss. At Guys and St. Thomas', we adopt a multidisciplinary approach. Patients attend

regular follow up led by a consultant urologist with a specialist interest in stone disease, supported by a renal physician, dietician, urologist and dedicated stone specialist nurses.

There is a wide variation in disease presentation that is not well understood and cannot be explained by either compliance with medical and dietary interventions, or differences in patient management. Predicting disease severity and managing patients expectantly is confounded by a paucity of validated methods to monitor disease activity and heterogeneity in how urinary dibasic amino acid levels are measured and reported in the literature. Furthermore, there are over 200 genetic mutations that have been described in literature, with poor correlation to the clinical phenotype. The genetic mutations in a UK population have not been previously characterised.

The objective of my research was threefold; to understand the factors that lead to severe disease, to investigate clinical markers of disease activity, and to understand the genetic mutations that cause the disease. I characterised the clinical phenotype of patients with cystinuria by prospectively collecting patient data from patients attending our specialist clinic. I also prospectively collected urine samples in clinic (early morning urine and clinic urine samples) for measurement of urinary dibasic amino acids levels and crystals. I investigated the utility of the dibasic amino acid levels and urinary crystals as clinical markers for disease activity by investigating the correlation between urinary dibasic amino acid levels, urinary crystals and clinical phenotype. Robust clinical

markers will be instrumental in measuring the severity of patient disease and efficacy of patient management.

I also characterised the genetic mutations that cause cystinuria in a UK population and investigated associations between genotype and clinical phenotype. I used computational protein modelling to explore the mechanisms underlying the genotype-phenotype associations. I believe a better understanding of genetics and how it influences clinical phenotype will result in a better understanding of the differences in disease presentation in patients. This will lead to improvements in management which can be tailored to the individual genotypic needs.

2 Challenges in managing patients with Cystinuria

2.1 Introduction

Due to a dysfunctional protein transporter of the dibasic amino acids, cystinuria patients are at risk of forming cystine stones. The clinical presentation varies; some patients will pass occasional stones and others will present frequently with colic requiring hospital admission, medical or surgical intervention. Patients with 'severe' disease are at significant risk of renal loss. The aims of follow up of patients with this condition are preventative and pre-emptive intervention on growing stones before they can cause colic and other stone-related complications.

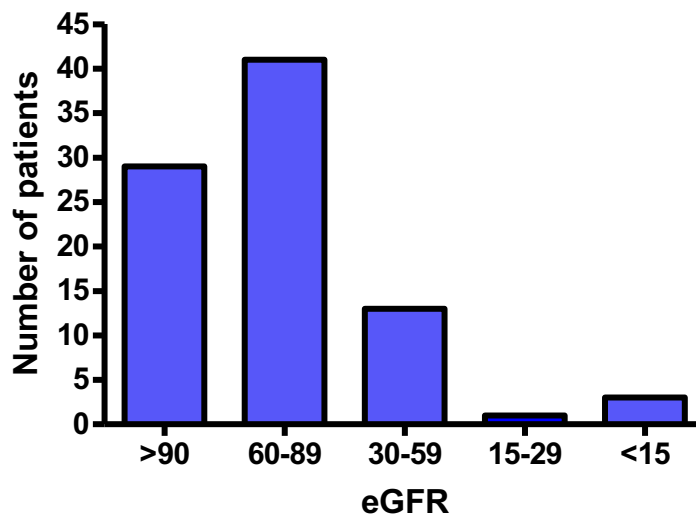


Figure 2.1 Estimated Glomerular Filtration Rate of our cystinuria cohort as measured by the Modification of Diet in Renal Disease equation adjusted for age and gender of patient.

The underlying pathophysiology of why some patients have clinically mild disease and others have more severe forms requiring frequent surgical intervention is unclear. It is not understood what triggers the disease to present and currently no guidelines on how patients should be monitored once they are diagnosed. Often, whether a patient has mild or severe disease is not clear until the patient has been observed for a number of years. Research in this condition however, is hampered by several factors; firstly, as a rare disease, patient numbers are few and most urologists may only see one or two cases of cystinuria in their lifetime. Secondly, we interfere with the natural history disease by preventative measures, pre-emptive management and treatment. Finally, there is currently no validated method to measure disease activity. The focus of my research is therefore to understand the factors that lead to disease, to investigate the markers of disease activity, and to understand the genetic mutations that cause cystinuria and how they might correlate with phenotype.

2.2 Specialist Multidisciplinary Cystinuria Clinic

The Guys and St Thomas' Hospital specialist Cystinuria clinic was established in August 2008 by a Consultant Endourologist (KT). Patients have a joint review with a Consultant Urology and Consultant Nephrologist, undergo an ultrasound scan by a specialist Uroradiologist and specialised review by a dietician. The optimum follow up schedule is unknown and unique and tailored to each patient. They are seen regularly from three monthly to annually depending on the severity of their disease. The frequency of follow up is dictated by the severity of

the individual patient's condition with a threefold aim; stone prevention, to capture new stone growth and intervene to prevent stone-related complications.



Figure 2.2 Map of the UK patients who attend our specialist clinic

At each clinic visit, patients bring in an early morning urine sample and a clinic sample for spot measurement of urinary dibasic amino acids and crystalluria. In particular, attention to hypertension, hypercholesterolaemia and diabetes mellitus are undertaken to optimize long-term renal function⁶. Patients are encouraged to take control of their disease and to do as much home monitoring as possible with regard to fluids, pH, diet, weight loss and blood pressure. Renal function and other blood tests are monitored regularly particularly for patients on thiol-binding drugs. Patients are consulted during their review as to whether they have had any stone episodes since their last visit. A stone episode is defined as the spontaneous passage of stones, an episode of acute colic with or without hospital attendance, or clinically significant stones that require intervention including ESWL, URS and PCNL. When choosing what intervention is required, patient factors are also taken into account; one may want to avoid an operation in a patient who has multiple comorbidities and may be at high risk for a general anaesthetic and attempt ESWL if appropriate, bypassing the need for a general anaesthetic in the first instance.

In practice, the frequency of follow up is heavily influenced by a patient's initial presentation, until sufficient time has passed and the individual's disease course becomes clearer. For example, a patient whose stone disease is dormant for several years and presents with large bilateral staghorn calculi would be considered to have a severe clinical phenotype and may be followed up more closely than a patient who presents initially with a single renal stone <1cm. Patients who present younger are also considered to have more severe disease than those who present at an older age in keeping with what we know of cystine

supersaturation⁶⁴. The practice of more frequent follow-up of more clinically severe patients and less frequent follow up in patients perceived to have a milder form of cystinuria creates a clinician-led selection bias. Furthermore, we try to balance the frequency of follow up against the disruption to patient's lives as many of our patients travel long distances to attend the clinic.

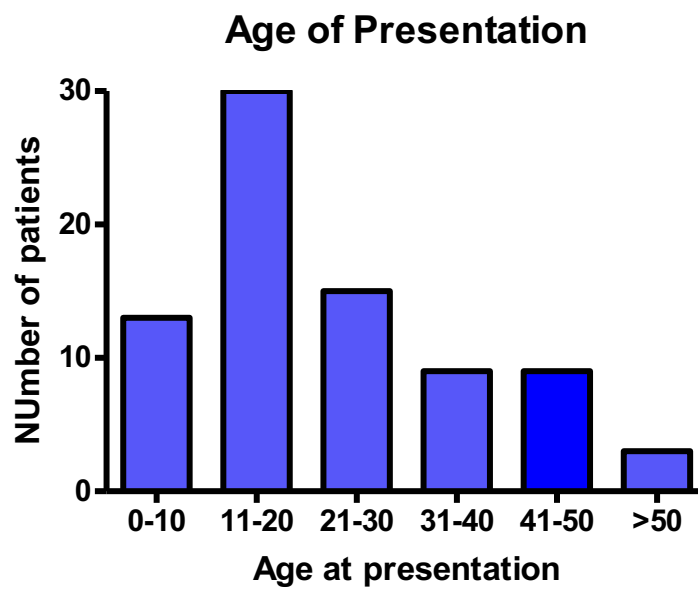


Figure 2.3 Age at presentation of patients with Cystinuria in our cohort (Adapted from Thomas, Wong et al 2014)

As we are screening siblings of patients who already have a diagnosis of cystinuria, we are building a unique cohort of people who have cystinuria mutations who at the point of diagnosis may be stone-free. We have the opportunity to monitor these people and learn about the natural course of the disease before these people even present with stone disease.

All patients attending clinic are invited to participate in our genetics study and consent to a peripheral blood sample taken for DNA sequencing and multi-ligand dependent probe amplification.

One of the main challenges in clinical research in cystinuria is that there is currently no validated measurement of disease severity. Given the high frequency of which some patients' experience stone episodes, patients recall of stone episodes can be unreliable. In addition, any clinical intervention including dietary or medical therapy can potentially alter the natural progression of the disease⁴⁸. Furthermore, there is a recognisable bias with more frequent follow up of patients with more frequent stone episodes or a larger stone burden.

A predictive clinical marker of stone growth and burden would therefore be particularly useful in the long-term management of patients with cystinuria. An optimal follow-up regimen could then be tailored to the disease burden of individual patients, ensuring that review is frequent enough not to miss significant stone formation, allowing pre-emptive intervention if necessary to prevent stone related complications. No such marker currently exists.

2.3 The utility of dibasic amino acids

Cystinuria is caused by an accumulation of urinary cystine. Traditionally, measurements of urinary cystine were taken over a 24-hour period. Normal urinary cystine level is between 0-100 μ mol/g creatinine in 24 hours⁹. Collection of 24-hour urine is cumbersome for patients particularly when they are traveling long distances to the clinic. Although 24-hour urinary cystine is useful diagnostically, it has not been shown whether it has any prognostic value. In our laboratory, we use 'spot urine' measurements, a validated and pragmatic approach to monitoring. Studies on the biochemical composition of spot urine demonstrates that it can accurately reflect 24 hour urine collection⁶⁵ however its use as a marker of disease activity has not previously been tested.

However, standard hospital laboratory assays measure urinary free cystine which does not account for the insoluble cystine present in crystal form. Furthermore, current assays do not distinguish between free cystine and cystine bound to thiol-containing drug complexes as used in the treatment of cystinuria. Therefore, the measured urinary cystine level is not a true reflection of total cystine. Coe et al have described a solid-phase assay to measure urinary cystine supersaturation and cystine capacity and have found this method to be reliable but it is currently not widely available limiting its use^{37,64}. Using this technique, patients who present at an older age exhibit a lower cystine supersaturation level than younger patients suggesting an age protective effect.^{65,66}

I examined the association between free urinary cystine measurement and patient's stone episodes. Furthermore, the clinical utility of other dibasic amino

acids (lysine, arginine and ornithine) which are also raised in cystinuria has not previously been investigated. As urinary cystine measurements are not a reflection of true levels, we hypothesized the measurement of the other dibasic amino acids may clinically be more useful.

2.4 The prognostic value of urinary crystals

Crystalluria is a necessary step in cystine stone formation. Daudon et al have previously shown in other types of stone disease, the presence of urinary crystals is predictive of stone recurrence⁶⁷. We hypothesize the measurement of urinary crystals in cystinuria may be useful clinically to predict stone episodes and can be used as a biomarker to determine stone formation.

We ask patients for a clinic urine and early morning urine sample with each clinic visit. Early morning urine has traditionally believed to be most reflective as it is the most concentrated urine collected in a 24-hour period due to overnight accumulation of crystals. We compared the utility of both early morning urine samples with a mid-morning clinic urine sample.

Prior studies on urinary crystalluria have used the Malassez counting chamber^{67,68}. We compared the utility of the Malassez counting chamber with conventional cytopsin methods which could potentially be automated.

2.5 Genetics

Historically, the classification of cystinuria was based on parental urinary phenotype. Type I is autosomal recessive; these patients have parents who are carriers, each with one mutated and one normal allele. The parents excrete normal amounts urinary cystine. Type II and III have parents who have varying levels of dibasic amino acids. In type II, parents excrete $>900\mu\text{mol/g}$ of creatinine, type III parents excrete $100\text{-}900\mu\text{mol/g}$ of creatinine. Type II and III cystinuria are therefore regarded as autosomal dominant with incomplete penetrance. Advances in molecular genetics have led to greater understanding of the underlying mutations that cause cystinuria. The old classification has been superseded by a newer classification where type A refers to all patients with SLC3A1 mutations which confer a type I phenotype. Type B refers to patients with SLC7A9 mutations which cause all three phenotypes. Type AB are patients with mutations in both³⁴.

Over 200 genetic mutations have been described in cystinuria worldwide. The mutations are diverse in both SLC3A1 and SLC7A9. 50% of patients with type A cystinuria will present with stone disease in their first decade of life and another 25% in their teenage years³⁴. Genotype-phenotype studies have been done showing no difference in clinical presentation between type A and type B disease¹⁰.

My thesis describes one of the first studies which look at mutations specific to a UK population. I characterised the genetic mutations that cause cystinuria and

investigated associations between genotype and clinical phenotype, using protein modelling to explore the underlying mechanisms.

3 The Utility of Dibasic Amino Acids

3.1 Introduction

Spot measurements of urinary cystine have been shown to accurately reflect 24-hour urine excretion, as described in chapter 2. Spot urinary cystine has been useful diagnostically but its prognostic value has yet to be determined. At the Guys and St Thomas' specialist cystinuria clinic, we collected spot urine at each patient clinic visit for analysis. I was interested in investigating the clinical utility of measuring urinary cystine as well as the other dibasic amino acids (lysine, arginine and ornithine).

There are various methods in which free unbound urinary cystine can be analysed including the use of cyanide-nitroprusside and spectrophotometry, Liquid Chromatography-tandem Mass Spectrometry (LC-MS), and High Performance Liquid Chromatography (HPLC). Not all techniques however are widely available in a hospital laboratory for diagnostic use⁶⁹. Our laboratory uses ion-exchange HPLC with post-column ninhydrin complex formation and detection of the coloured compound produced at 570nm. The expected free urinary cystine in a patient without disease is in the range of 4-15umol/mmol Cr. In a patient with cystinuria, urine concentration reach levels of around 300umol/mmol Cr.

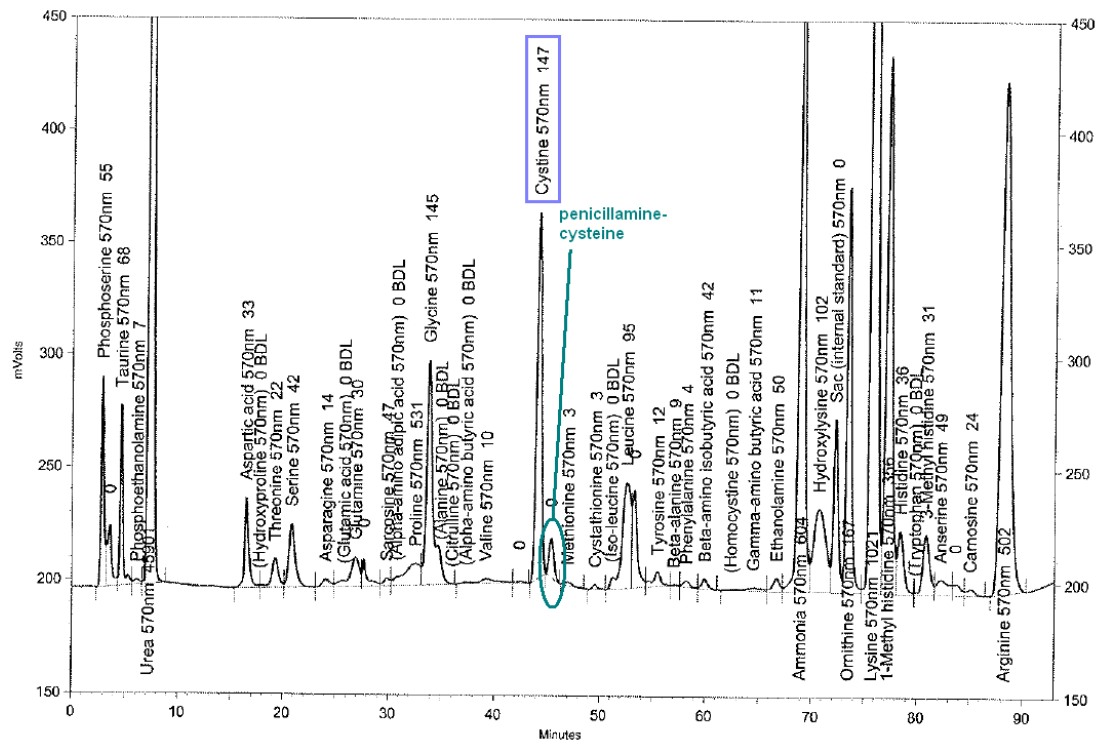


Figure 3.1 Figure showing a biochrom chromatogram of a cystinuria patient on penicillamine (Adapted from Mozley et al). Note a penicillamine –cysteine peak can also be seen.

This Biochrom technique however requires acidification of urine samples for analysis to a pH <2 which potentially can alter the solubility of cystine in the sample^{18,70,71}. Dolin et al described a technique that involves alkalinisation of urinary samples with sodium bicarbonate to a pH >9 to ensure all the cystine in the sample is solubilised⁷².

We performed a small study in collaboration with the University of Southampton on 14 patients to assess the method of cystine quantification by comparing our Biochrom technique with the Biochrom at the University of Southampton in addition to an internally validated LC-MS method. I prepared the samples for analysis under the guidance of Dr Charles Turner. Dr Erin Mozley performed the sample analysis through the Biochrom and LCMS. We performed a sub-group analysis comparing alkalinised samples using a modified Dolin technique and non-alkalinised samples using both methods.

| GSTS Biochrom ¹ | | | Cr ^B mmol/L | | | Southampton Biochrom ² | | | | LC-MS ² | | | | pH | | |
|----------------------------|---------------------------|-----------------------------|---------------------------|---------------------------|-----------------------------|-----------------------------------|-------------------|-----------------------------|-------------------------|--------------------|------------------|------------|-----|---------|------|-----|
| No. | Cr ^A mmol/L | Non-alkalinised /mmol Cr | Non-alkalinised /L | Cr ^B mmol/L | Non-alkalinised /mmol Cr | Alkalinised /mmol Cr | Alkalinised /L | Non-alkalinised /mmol Cr | Alkalinised /mmol Cr | Alkalinised /L | Acid /mmol Cr | Acid /L | Alk | Non-alk | | |
| 1 | 3.7 | 250 | 925 | 9.3 | 243 | 2260 | 256 | 2381 | 216 | 2012 | 246 | 2286 | 634 | 2344 | 11 | 6.5 |
| 2 | 5.9 | 176 | 1038 | 3.1 | 166 | 515 | 167 | 518 | 194 | 602 | 188 | 584 | 318 | 1876 | 11 | 7 |
| 3 | 4.7 | 191 | 898 | 1.4 | 236 | 330 | 230 | 322 | 255 | 357 | 259 | 362 | 120 | 562 | 11.5 | 7 |
| 4 | 11.0 | 145 | 1595 | 5.6 | 153 | 857 | 151 | 846 | 165 | 926 | 189 | 1056 | 117 | 1283 | 11 | 7 |
| 5 | 7.3 | 221 | 1613 | 5.1 | 210 | 1071 | 200 | 1020 | 202 | 1031 | 185 | 944 | 197 | 1435 | 11 | 6 |
| 6 | 2.0 | 261 | 522 | 3.6 | 268 | 965 | 268 | 965 | 297 | 1070 | 276 | 993 | 805 | 1609 | 11.5 | 7 |
| 7 | 6.5 | 297 | 1931 | 6.4 | 276 | 1766 | 275 | 1760 | 289 | 1850 | 255 | 1629 | 316 | 2052 | 12 | 7 |
| 8 | 7.8 | 130 | 1014 | 6.4 | 202 | 1293 | 59 | 378 | 174 | 1112 | 130 | 835 | 171 | 1337 | 12 | 5.5 |
| 9 | 5.7 | 87 | 496 | 4.5 | 151 | 680 | 163 | 734 | 181 | 813 | 164 | 739 | 175 | 998 | 12 | 7 |
| 10 | 6.1 | | | 5.2 | 418 | 2174 | 282 | 1466 | 332 | 1727 | 296 | 1538 | 261 | 1593 | 12 | 5.5 |
| 11 | 3.5 | | | 2.9 | 174 | 505 | 147 | 426 | 188 | 544 | 202 | 587 | 182 | 638 | 11.5 | 7 |
| 12 | 14.9 | | | 12.7 | 126 | 1600 | 238 | 3023 | 130 | 1656 | 247 | 3134 | 105 | 1560 | 12 | 7 |
| 13 | 17.1 | | | 14.7 | 218 | 3205 | 184 | 2705 | 212 | 3119 | 195 | 2868 | 173 | 2957 | 12 | 8 |
| 14 | 8.5 | | | 7.4 | 123 | 910 | 318 | 2353 | 110 | 813 | 300 | 2220 | 157 | 1334 | 11.5 | 7 |
| N1 | 15.2 | 20 | 304 | 12.6 | | | | | 22 | 280 | 21 | 267 | 21 | 313 | 11.5 | 2 |
| N2 | 0.5 | 0 | 0 | 0.3 | | | | | 0 | 0 | 0 | 0 | 0 | 0 | 11 | 7 |
| N3 | 1.1 | 6 | 7 | 0.5 | | | | | 4 | 2 | 12 | 6 | 3 | 3 | 11.5 | 5.5 |
| N4 | 1.2 | 10 | 12 | 0.8 | | | | | 13 | 10 | 16 | 13 | | | 11.5 | 8 |
| S | | | | 10.4 | 8 | 83 | 7 | 73 | | | | | 2 | 22 | 12 | 6.5 |

Figure 3.2 Summary of the results comparing the different methods of cystine quantification in the two laboratories (Mozley et al). N= 14 patients with cystinuria. N1-4 represents 4 normal patients. S represents stock urine.

There were no significant differences between the samples measured in our laboratory of either the non-alkalinised ($P = 0.1976$, $r^2 = 0.7707$) or alkalinised ($P = 0.9309$, $r^2 = 0.6782$) samples measured in Southampton and there was good correlation between the Biochrom method with the LC-MS method; our Biochrom ($r^2 = 0.8769$ and $r^2 = 0.8750$) and Southampton Biochrom ($r^2 = 0.8728$ and $r^2 = 0.0789$).

When assessing the pre-alkalinised and non-alkalinised samples, there was no statistical difference found and the correlation using both instruments was poor (LC-MS $r^2 = 0.6565$, Southampton Biochrom $r^2 = 0.3353$). It was observed in the two patients (number 12 and 14) with the highest cystine concentrations had a higher concentration in the alkalinised samples compared with the non-alkalinised samples although this was not statistically validated. We hypothesize from this that alkalinisation may affect cystine concentration in highly concentrated samples although we have not formally tested this. We continue to use the Biochrom method for the quantification of urinary cystine in our laboratory.

We evaluated the prognostic value of spot urinary measurements of cystine, lysine, arginine and ornithine with respect to stone formation in patients with cystinuria. We hypothesized that stone episodes would correspond to a peak in the levels of urinary dibasic amino acids.

3.2 Patients and Methods

Data was collected between August 2008 to February 2014. A sample of urine was collected at each patient visit and sent for measurement of urinary dibasic amino acid measured using the Biochrom method (Biochrom 30 Amino Acid Analyzer). Amino acid values were measured as millimoles per mole of creatinine (umol/mmol Cr). Follow up ranged from three to twelve monthly. At each clinic visit, patients had a renal ultrasound scan for detection of stones. Patients with less than one year radiological follow up were excluded.

New stone formation (NSF) was defined as evidence of new stones or an increase in size of existing stones seen on imaging as compared with baseline imaging. Thus, first clinic visit for each patient with at least six months follow up was used. Imaging was analysed for NSF at the time of clinic, three and six months prior to clinic, three and six months post clinic.

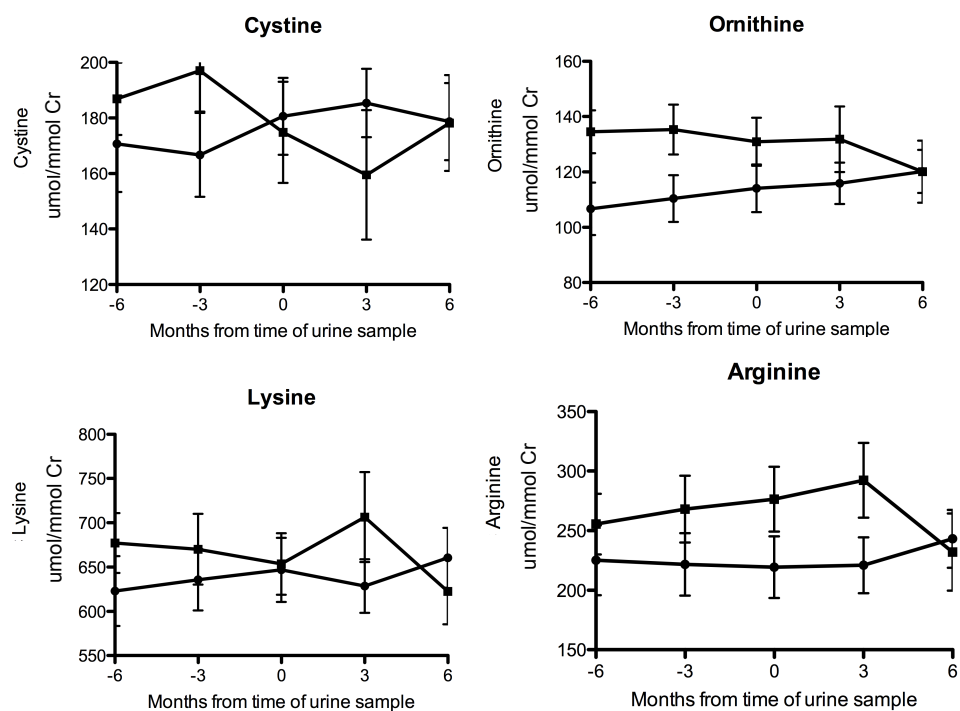
The levels of the urinary dibasic amino acids were compared between patients who had NSF and those with stable disease at each of the time points. The levels of the urinary dibasic amino acids were also compared between those with stone formation over a year and those without. We used D'Agostino-Pearson test to test the normality of our data and Mann U Whitney test was used to compare with levels between the two groups. A p value <0.05 was considered significant. Analysis was performed using Graph Pad Prism 5.0.

3.3 Results

64 patients were included in the study. 33 additional patients were excluded as they had a follow up of less than one year. These were patients who were either recently referred with less than one year follow up data or seen as a one-off review with routine imaging performed at their local hospital. There were 32 male and 32 female patients with a median age of 42 years (range 7-73). 13 patients were taking a thiol at the time of the urine sample (4 taking penicillamine, 9 taking tiopronin).

39 patients (61%) had radiological evidence of new stone formation over the one-year period. 11 of the 13 patients taking a thiol (85%) had evidence of new stone formation during this period.

The levels of each of the urinary dibasic amino acid for patients with new stone formation at the time of clinic, within three months and six months inclusive are represented below.



● No new stone formation
 ■ New stone formation

Figure 3.3 The levels of each of the urinary dibasic amino acid (cystine, lysine, arginine and ornithine) were compared between those new stone formation and those with stable disease (Pardy et al 2011⁴).

There was no statistically significant relationship between the levels of cystine, lysine, arginine and ornithine at each time point. There was a statistically significant higher level of ornithine in patients with NSF than in patients with stable disease at three and six months prior to stones seen on ultrasound.

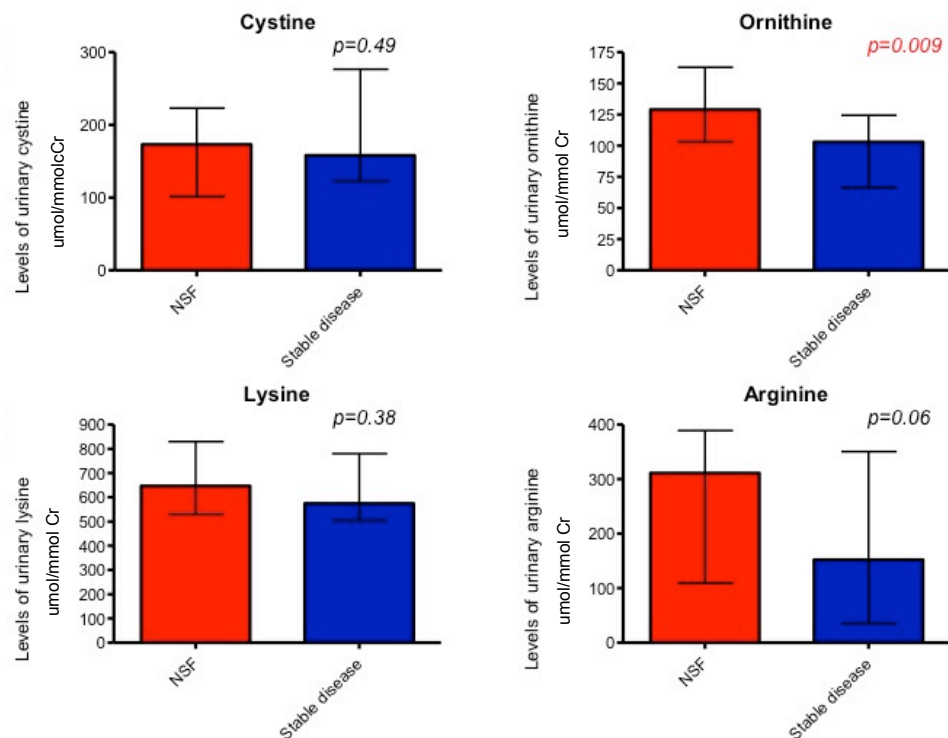


Figure 3.4 Comparison of levels of urinary dibasic amino acid (cystine, lysine, arginine, ornithine) between patients with new stone formation and those with stable disease at time of ultrasound imaging time = 0 (adapted from Pardy et al 2011⁴). Patients with evidence of new stone formation defined as new stones or stone growth seen on imaging compared with baseline had a statistically significant higher level of urinary ornithine ($p=0.009$). Patients with NSF appear to have higher levels of urinary arginine than patients with stable disease, but this was not statistically significant. There were no differences in the levels of urinary cystine or lysine between NSF and those with stable disease.

3.3.1 Subgroup analysis of patients taking thiol medication

A subgroup analysis was performed on patients taking thiol medication. There was no association between the levels of cystine, lysine and arginine and new stone formation at any time point. The association between urinary ornithine and new stone formation at three and six months prior to the urine sample was maintained, but no association was shown at 0, 3 and 6 months after the urine sample. Only two patients taking thiol medication had no evidence of stone formation over a one-year period prohibiting any statistical analysis.

3.4 Discussion

Cystinuria is a result of the accumulation of urinary cystine. Therefore, it would seem reasonable to hypothesize that the higher the levels of urinary cystine, the greater the likelihood of stone formation. In this study, we investigated the utility of urinary cystine and the other dibasic amino acids as clinical markers for predicting stone formation. Investigating the prognostic utility of spot urinary cystine and the other non-cystine dibasic amino acids has never previously been examined.

Our results do not demonstrate a correlation between urinary cystine levels and new stone growth. There are several possible explanations to account for this. Firstly, there are previously described limitations in urinary cystine measurement; it exists in soluble and insoluble form and only the soluble form is

measured, the cystine solubility is affected by factors such as pH and temperature, and the majority of cystine assays do not distinguish between free urinary cystine and cystine bound to thiol-binding complexes, both of which are soluble.

In addition, all cystine quantification methods require the acidification of the samples at some stage. In preparation for ion-exchange chromatography, the urine is diluted with a loading buffer of pH 2. Carla et al showed in in vitro studies that the solubility of cystine demonstrates a U-shaped pattern, that is, at a pH of less than 2, the solubility of cystine increases giving rise to the possibility that the effects of acidification may be of minimal consequence⁷¹. Dolin et al have described a method of increasing the solubility of cystine in solution by the addition of sodium bicarbonate to a pH of 9⁷². We have not been able to replicate this in our laboratory and have found the presence of cystine crystals in urine despite the addition of sodium bicarbonate to those samples.

In our prior study, we compared the measurement of urinary cystine using two different methods; HPLC and LCMS with alkalinised and non-alkalinised samples. The results demonstrate no difference in measurement of urinary cystine by the two methods and no difference in alkalinised versus non-alkalinised samples. Two patients however did demonstrate higher concentrations in alkalinised samples and further evaluation of this is needed. Although methods of cystine supersaturation have been described in literature as having prognostic value, currently, the methods require long sample preparation

and dangerous reagents which may not be suitable for routine testing in a standard hospital diagnostic laboratory¹⁸.

We evaluated the prognostic value of the other urinary dibasic amino acid on stone formation. There was no statistically significant difference in levels of urinary lysine in patients with NSF compared to those with stable disease. There is some evidence to suggest that there may be a separate transport system separating lysine from the other dibasic amino acids⁷³⁻⁷⁵. There are several differences in the handling of urinary cystine and lysine as highlighted in studies on rats⁷⁶. Cystine and lysine share a high-affinity system, however a low-affinity system exist which is unshared. In cortical slices, cystine transport is sodium dependent whereas lysine is sodium independent. Lysine is transported against a concentration gradient whereas cystine is not⁷⁶.

There was a significant difference between the levels of urinary ornithine in patients with evidence of NSF at 3 and 6 months prior to production of urine sample ($p=0.021$ and $p=0.04$ respectively). There was however no association in levels of ornithine at time of urine sample, 3 and 6 months post urine sample. This suggests perhaps the measurement of urinary ornithine may reflect a propensity for stone formation with a lag period of 3 – 6 months. This is potentially useful in predicting which patients are likely to form stones. The use is limited however to patients who are on lifestyle modification and dietary therapy only, and may not be useful in patients on thiol medication as thiol-binding medications specifically target urinary cystine and we do not know of any effect on urinary ornithine.

Our results show that although NSF have higher levels of urinary arginine than patients with stable disease, this was not statistically significant ($p=0.06$). This may be limited by a small sample size. We also observed that individual levels of urinary dibasic amino acids vary; some patients have proportionally higher levels of one urinary dibasic amino acid to another. More research is needed to understand the interactions between the individual dibasic amino acids.

Other methods to quantify urinary cystine including colorimetric methods such as cyanide-nitroprusside and spectrophotometry, and other forms of HPLC with or without derivatisation were not explored in this study. Furthermore, we still need a better understanding of what factor facilitate crystal formation and recent invitro studies suggest that inosine, vanillylmandelic acid and guanosine may have a role⁷⁷. Understanding how cystine is handled by the kidneys and the factors that promote stone formation can offer potential insight into new targets for therapy in cystinuria.

3.5 Conclusion

The quantification of spot urinary cystine does not appear to be a useful disease marker in the monitoring of disease activity in patients with cystinuria. Urinary ornithine and possibly arginine may reflect a propensity to stone formation however the use of this is limited to patients on lifestyle and dietary therapy only as lifestyle and dietary measures aim to affect cystine levels only. In our experiment, we have not been able to successfully replicate the dissolution of

cystine crystals by the addition of sodium bicarbonate to the samples as described by Dolin et al⁷². In the next chapter, we explore the utility of crystalluria as a clinical marker for disease activity.

4 The Diagnostic value of Crystalluria

4.1 Introduction

A predictive clinical marker of stone growth and burden would be particularly useful in the long-term management of patients with cystinuria. There are currently no guidelines regarding the frequency to which these patients should be seen in clinic. An optimal follow-up regimen would be tailored to the disease burden of individual patients, ensuring that review is frequent enough not to miss significant stone formation, allowing pre-emptive intervention if necessary to prevent stone related complications. If a urine sample with crystals can predict the presence of stone formation or new stone growth, it may be a useful adjunct or even a substitute to frequent imaging and clinic visits which compromise patient's lifestyle and increase cost of healthcare services.

The measurement of free urinary cystine alone is not an accurate reflection of total urinary cystine as it exists in both soluble and insoluble form and is affected by factors such as urine volume and pH^{18,64,70}. Furthermore, current cystine assays do not distinguish between free urinary cystine and the cystine bound to thiol-drug complexes which are used in the treatment of cystinuria. Hence, no correlation has been found between the levels of urinary cystine and stone episodes⁴. Given that urinary crystalluria is a necessary step in stone formation, we hypothesized the measurement of urinary crystalluria may be useful clinically to predict stone episodes. If the presence of crystalluria can predict stone formation, this could be used as a point of care test and replace the need for frequent clinic attendance and ultrasound imaging. However, the correlation

between urinary crystalluria and presence of stones or new stone growth needs to be robust enough not to miss significant stone episodes. Stone formers have previously been demonstrated to exhibit a higher cystine crystal volume than non-stone formers⁶⁸, however, the technique described is not readily available in standard hospital diagnostic laboratories, and could not readily be adapted as a point of care test. This is the first study to investigate whether urinary crystalluria can be reliably used as a diagnostic marker for the presence of stone disease and new stone growth.

In this study we aim to assess the feasibility of using crystalluria as a clinical marker for the presence of stones or new stone growth. However, if crystalluria is to be routinely used as a marker for disease activity in cystinuria, then the association between urinary crystals and new stone growth needs to be robust.

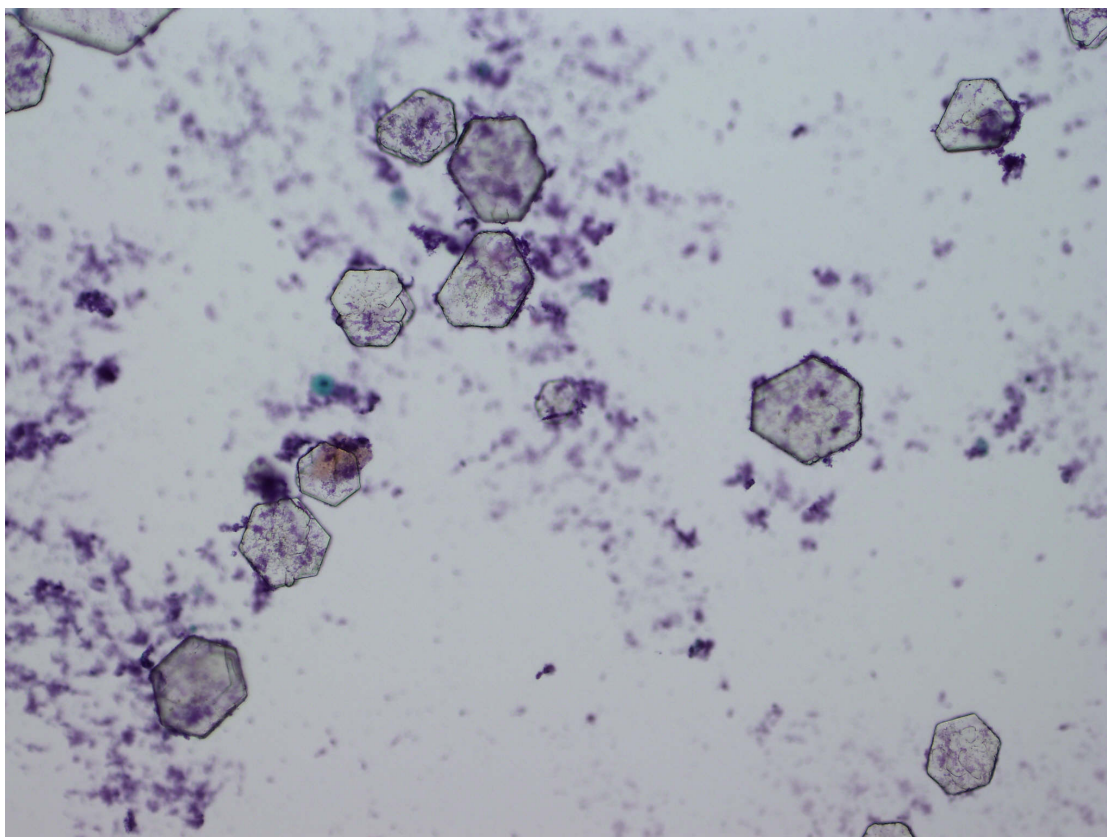


Figure 4.1 Urinary cystine crystals (Courtesy of Cystinuria UK)

Urinary crystalluria has been shown to be highly predictive of stone recurrence in calcium stone formers⁶⁷. Daudon et al have shown that the volume of crystals (V_{crys} ; defined as $0.65 \times N \times L^2 \times T$ where N =number of cystine crystals per mm^3 , L =average length of crystals and T =average thickness of crystals) is significantly higher in patients with recurrent stone formation⁶⁸. This technique, however, is time consuming and not readily available in a standard hospital diagnostic laboratory. In contrast, the number of urinary crystals can be easily determined. However, it is not known whether the number of urinary crystals in cystinuria is prognostic with respect to stone formation⁷⁸. Prior studies on urinary crystalluria have used the Malassez counting chamber^{67,68}. We were keen to compare the utility of the Malassez counting chamber with cytopsin methods which could potentially be automated. Previous studies measuring crystalluria have used early morning urine (EMU) samples, as they were considered to represent the most saturated sample in a 24-hour period^{22,24,26,79}. We sought to compare the predictive value of crystalluria in EMU with a mid-morning Clinic Urine (CU) sample, which we feel may be more readily applied in clinical practice.

We investigated the efficacy of crystalluria as a clinical marker for disease activity in patients with cystinuria. Specifically, we looked at:

1. The association between the presence of urinary crystals in EMU and CU and samples with the presence of stones and new stone growth
2. The utility of the Malassez counting chamber with cytopsin methods for measurement of urinary crystalluria
3. The correlation between the number of urinary crystals and presence of stones and new stone growth

4.2 Materials and Methods

At each clinic visit, patients were asked to bring an EMU sample and to provide a CU sample for measurement of urinary crystals. They all underwent an ultrasound scan by our urologist at the same consultation. All patients included in the analysis had a least one previous baseline ultrasound for comparison. New stone growth (NSG) was defined as an increase in stone size or new stones reported on ultrasound scan as compared with baseline ultrasound. Stable disease include patients who had no stones on their scan or stones that were present on their previous ultrasound scan and remain stable in size. To avoid patient bias, only the first urine sample for each patient was analysed. Samples were excluded if it was the patient's first visit as no old scan was available for comparison, if they had bladder stones or stents in-situ, and where the ultrasound scan was inconclusive.

The majority of the urine samples were processed on the same day in the hospital diagnostic laboratory. Samples that could not be processed on the same day were kept in a fridge at 4 degrees Celsius and processed the next available day. Sample preparation and counting methods were performed by Dr Soma Pillay (Consultant Cytopathology), Dr Ashish Chandra (Consultant Cytopathologist) and their team. The samples were first gently mixed using a rotamixer. Neat urine was aspirated into a capillary tube, allowed to diffuse onto the Malassez cell counting chamber of 0.2mm depth (CML, Nemours, France), and examined

by light microscopy. The number of crystals were counted on the entire chamber and the results expressed as number of crystals per mm³.

The number of urinary crystals for each sample was also determined using cytopsin cytocentrifuge technique. Again, the samples were first gently mixed using a rotamix. A standard cytopsin protocol was used; 0.25µl of neat urine from each sample was spun at 600g for five minutes, then placed on a standard microscopy slide and examined using a standard light microscope (Olympus BX53).

Where the sample tested had more than 1000 crystals per mm³, the crystals formed aggregates and it was impossible to count the crystals individually. It was therefore recorded as >1000. For the analysis, the cut-off value was taken to be 1000. The correlation between the presence of crystals and presence of stones seen on ultrasound imaging or new stone growth was compared using the Chi square test. ROC analysis was performed to assess the utility of urinary crystals as a diagnostic test for the presence of stones and new stone growth. We used D'Agostino-Pearson test to test the normality of our data and Mann-U Whitney was used to compare the number of crystals between patients with stone(s) or new stone growth and those with no stones or stable disease. A p value <0.05 was considered significant. Statistical analyses were performed using SPSS (IBM, Version 22.0) and ROC analyses were performed using MedCalc software⁸⁰ (MedCalc software Version 13.2.2, Belgium).

4.3 Results

63 paired patients samples (EMU & CU) were analysed between 1st of Aug 2010 and 31st of July 2012. To avoid patient sample bias, only the first paired sample for all patients was analysed as including all urine samples would bias the analysis towards patients who were seen more frequently in clinic and therefore provided more urine samples. These are potentially patients with more severe disease and more likely to have urinary crystals and stones on ultrasound.

| Patient Cohort | Frequency |
|---|--------------------|
| No of patients | 63 |
| Age at Presentation | 23 |
| Age Range | 9 mths to 61 yrs |
| M:F ratio | 1.25:1 |
| No of patients with stones seen on US | 31 |
| No of patients with new stone growth | 27 |
| Total number of samples | 126 |
| <u>No of samples with crystals present</u> | 56 |
| Stones vs no stones on US | 35(63%) vs 21(37%) |
| New stone growth vs stable disease on US | 27(48%) vs 29(52%) |
| <u>No of samples without crystals</u> | 70 |
| Stones vs no stones on US | 26(37%) vs 44(63%) |
| New stone growth vs stable disease on US | 17(24%) vs 54(77%) |
| <u>No of samples with >1000 crystals</u> | 14 |
| Stones vs no stones on US | 11(79%) vs 3(21%) |
| New stone growth vs stable disease on US | 7(50%) vs 7(50%) |

Table 4.1 Patient demographics and sample analysis. Subgroup analysis of samples with crystals present, samples without crystals and samples with more than 1000 crystals.

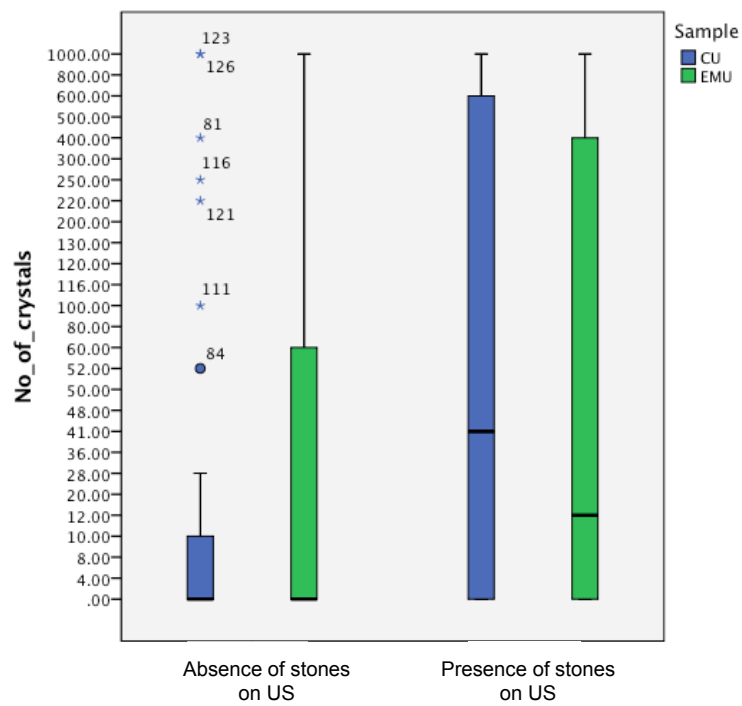
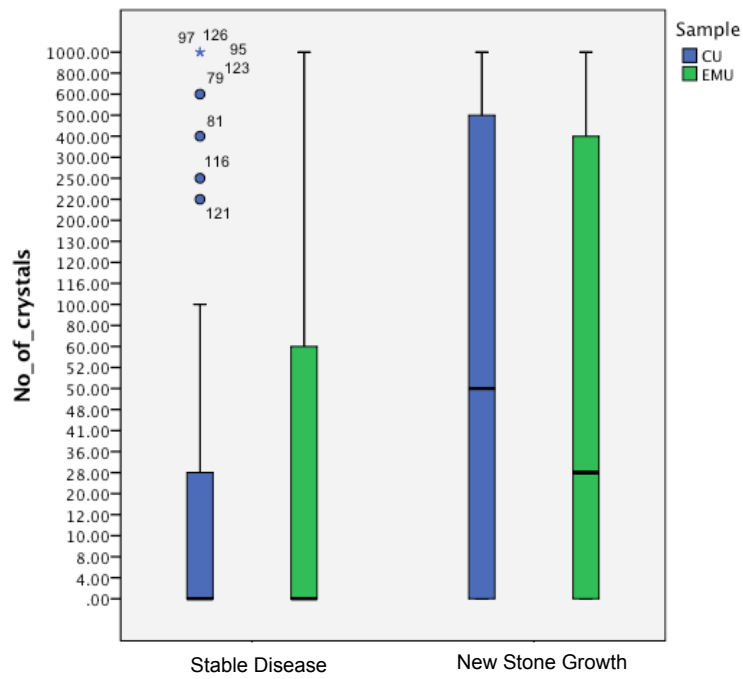


Figure 4.2 The number of crystals associated with EMU and CU samples.

4.3.1 Pilot study comparing the Malassez counting chamber and cytopsin methods

To investigate the optimum method of quantifying crystalluria, all samples were initially analysed using both the Malassez counting chamber method and the cytopsin method. Data was obtained from 40 patients. There was no statistically significant difference between the presence of urinary crystals and presence or absence of stones on ultrasound nor for new stone growth (NSG) versus stable disease for EMU using both methods using the chi-square test (stones, Malassez chi-square test =2.71, d.f.=1, p=0.10, Cytopsin chi-square test=1.52, d.f.=1, p=0.22; NSG, Malassez Chi-square test=0.89, d.f.=1, p=0.35, Cytopsin chi-square test =1.15, d.f.=1, p=0.28). When comparing the CU samples however, there was a statistically significant difference between the presence of crystals in patients with stones detected on ultrasound compared to patients with no stones using both methods (Malassez chi-square test=5.65, d.f.=1, p=0.02, Cytopsin chi-square test=6.42, d.f.=1, p=0.01). There was also a significant difference when comparing the presence of crystals between patients with NSG on ultrasound compared with patients with stable disease using cytopsin but not the Malassez counting chamber (NSG Malassez chi-square test=2.88, d.f.=1, p=0.09, Cytopsin chi-square test=5.18, d.f.=1, p=0.02). The area under the ROC was highest for clinic urine samples analysed by the cytopsin method (AUC 0.70; 95% CI 0.53 to 0.84)⁸¹.

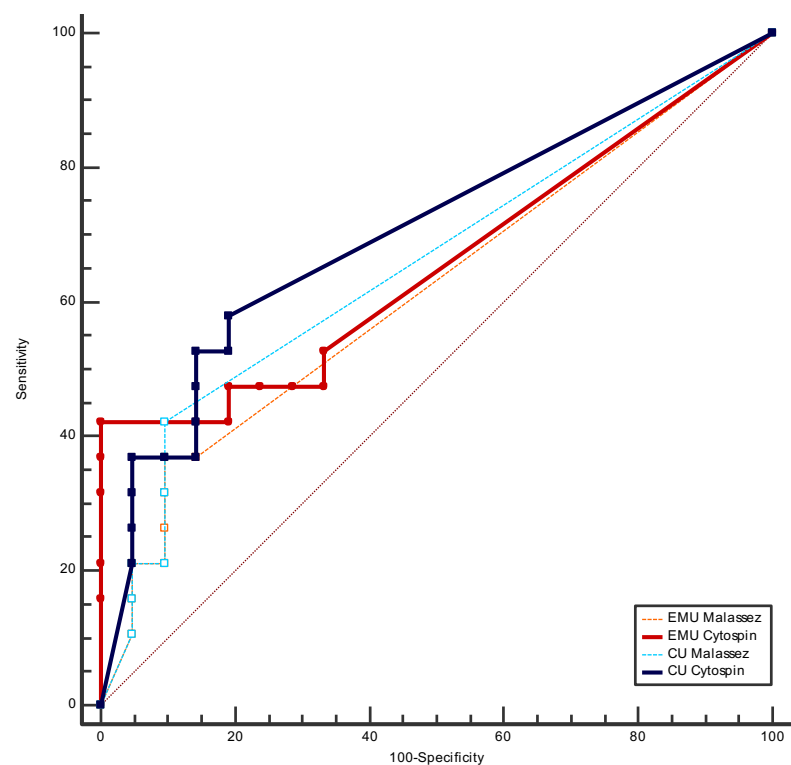


Figure 4.3 ROC curve of number of crystals in determining presence of stone(s) using both cytospin and Malassez counting chamber methods

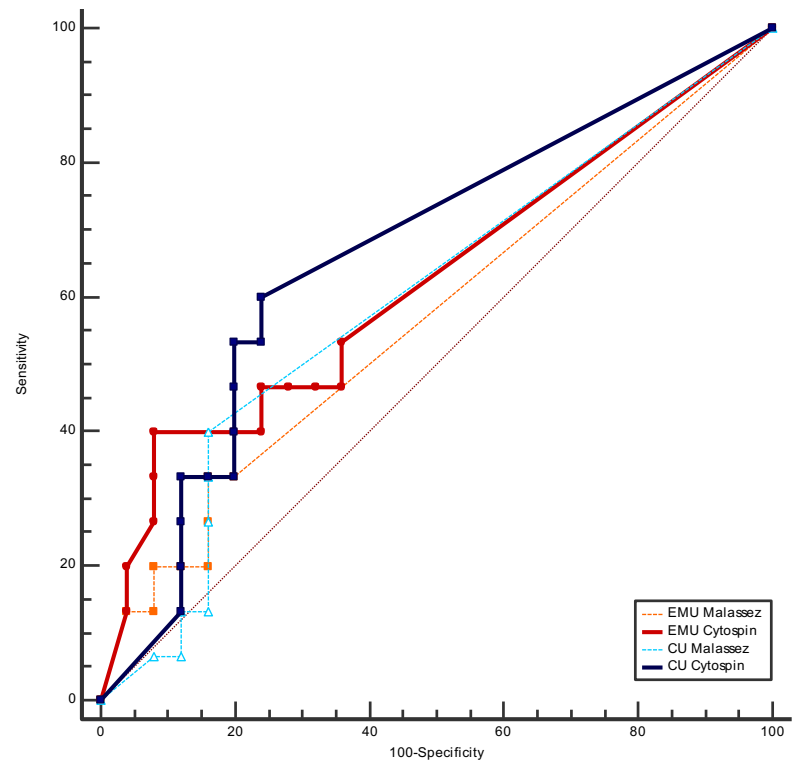
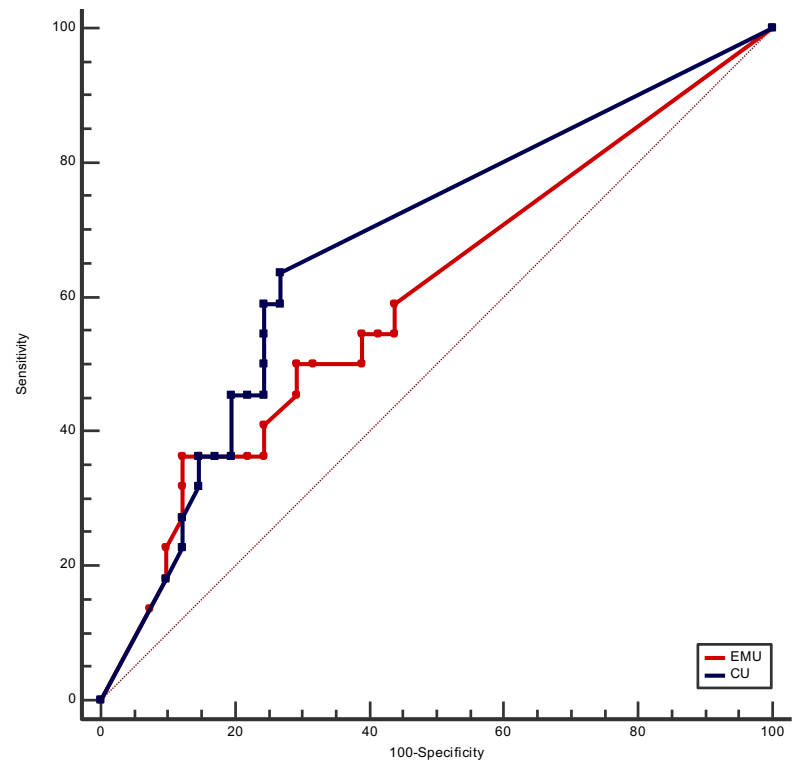


Figure 4.4 ROC curve of number of crystals in determining new stone growth using both cytopsin and Malassez counting chamber methods

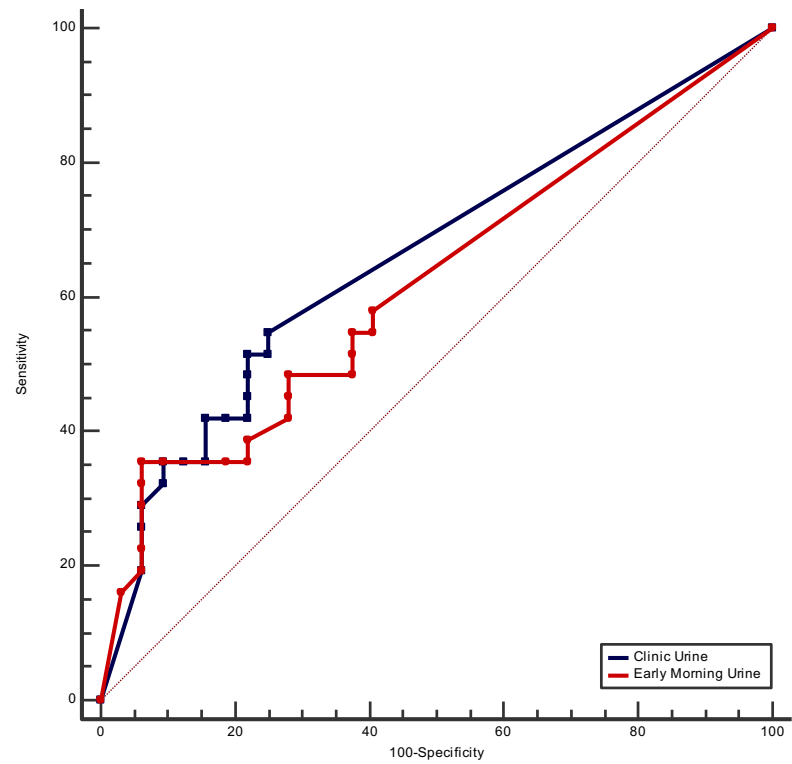
4.3.2 Comparison of EMU with CU samples

The use of the Malassez counting chamber was abandoned after our pilot data suggested that it was less effective than cytopsin (see previous section). When comparing EMU samples, there was no association between the presence of crystalluria in patients with stones seen on ultrasound imaging and those without (chi-square test=1.92 d.f.1 p=0.17). There was also no association between the presence of urinary crystals with new stone growth (chi-square test=1.32, d.f.=1 p=0.25). When comparing the CU samples however, there was a statistically significant difference between the presence of crystals seen in patients with stones seen on ultrasound imaging and those without (chi-square test=5.86 d.f.=1 p=0.02). The likelihood ratio was 5.96, d.f.=1 (p=0.02). There was a statistically significant difference between the presence of crystals seen in patients with NSG compared to those without (chi-square test=8.10 d.f.=1 p=0.004). The likelihood ratio was 8.11 d.f.=1 (p=0.004).



| | AUC | SE | 95% CI |
|---------------------|------|------|--------------|
| Clinic Urine | 0.66 | 0.06 | 0.53 to 0.78 |
| Early Morning Urine | 0.62 | 0.07 | 0.49 to 0.74 |

Figure 4.5 ROC curve comparing EMU and CU in determining the presence of stone(s)



| | AUC | SE | 95% CI |
|---------------------|------|------|--------------|
| Clinic Urine | 0.67 | 0.07 | 0.54 to 0.79 |
| Early Morning urine | 0.60 | 0.07 | 0.47 to 0.72 |

Figure 4.6 ROC curve comparing EMU and CU in determining new stone growth

Receiver operator curves for EMU and CU for all 63 patients are seen above. The area under the curve for crystals and presence of stones for CU samples was 0.661 (95%CI 0.53 to 0.78). The sensitivity and specificity was 56.7% (95% CI 37.4-74.5%) and 75.8% (95% CI 57.7-88.9%) respectively. The positive predictive value (PPV) was 68% (95%CI 46.5-85.1%) and negative predictive value (NPV) was 65.8% (95%CI 48.7 – 80.4%). The AUC for crystals and new stone growth for CU samples was 0.67 95%CI 0.54 to 0.79. The sensitivity and specificity of the presence of crystals in CU samples to predict new stone growth were 56% (95%CI 34.9 to 75.6%) and 80.5% (95%CI 65.1 to 91.2%) The PPV and NPV were 63.6% (95%CI 40.7 to 82.8%) and 75% (95%CI 57.7 to 86.8%). The sensitivity and specificity of the presence of crystals in CU samples to predict new stone growth was 59.09% (95%CI 36.4 to 79.3) and 75.61% (95%CI 59.7 to 87.6%) respectively at a level of >28 crystals as calculated by the optimal criterion.

There was a statistically significant difference in the number of urinary crystals in patients with stones compared with those without for CU samples (Mann-U Whitney; median=41, IQR=600 vs median=0, IQR 21, p=0.01). There was a statistical difference between the number of crystals (49, IQR 525 vs 0, IQR 40, p=0.01) between patients with new stone growth and patients with stable disease for CU samples. There was no difference between the number of crystals between patients with stones and those without for EMU samples (median=12 IQR=400 vs median=0, IQR=60, p=0.08). Likewise, there was no difference between patients with new stone growth and stable disease for EMU samples (median=24, IQR=450 vs median=0, IQR=88, p = 0.15).

4.4 Discussion

There is no reliable marker of disease activity currently available to clinicians managing patients with cystinuria³⁷. Our results show an association between the presence of crystalluria and presence of stones for CU samples but the correlation is not robust enough for the use of crystals as a single diagnostic test.

Previous studies have used the Malassez counting chamber for the measurement of urinary crystals in other types of stone disease and also in the measurement of cystine crystals volume^{67,68}. We compared the Malassez counting chamber with cytospin and found that cytospin yielded a higher area under the curve over the Malassez counting chamber, particularly for CU samples. We postulate that this was because the Malassez counting chamber does not allow large aggregates of crystals to fit into the chamber and were discounted from sampling potentially leading to an underestimation of the number of crystals, particularly in heavily saturated samples. The use of the Malassez counting chamber was subsequently abandoned in favour of the cytospin method.

Previously, the EMU was presumed to be the most reliable sample for measurement of crystalluria as it represents the most saturated sample during a 24-hour period due to overnight accumulation of urinary sediment. EMU samples have been used in studies of crystalluria for other types of stone disease such as calcium composite stones. Our results show that there was a higher correlation between the presence of urinary crystals and presence of stones in CU compared with EMU samples. We speculate that due to the high concentrations

of crystals expected in all cystinuria patients, the concentrated EMU may be an oversaturated sample that does not accurately differentiate between patients with high and low disease activity.

A patient with CU crystals is four times more likely than a patient without crystals to have stones on ultrasound imaging (OR 4.087 95%CI 1.395 to 11.97) and five times more likely to have new stone growth (OR 5.250 95%CI 1.739 to 15.85). 37% (26/70) of samples with no crystals had stones on ultrasound imaging. One possible explanation is that the fraction of urine analysed may not have adequately sampled crystals that may be present. However, this demonstrates that although there was a strong association between the presence of crystals and stones, the absence of crystals does not necessarily exclude the presence of stones. The overall NPV is 65.8% (95%CI 48.7 to 80.4%).

Furthermore, 11% (14/126) samples had over 1000 crystals per mm³. Where the sample tested had more than 1000 crystals per mm³, the crystals formed aggregates and it was impossible to count the crystals individually. It was therefore recorded as >1000. For the analysis, the value was taken to be 1000. This may decrease the sensitivity of the results and may partially account for the lack of statistical significance in the EMU analyses. The NPV of more samples with more than 1000 per mm³ is 56.4% (95%CI 42.3 to 69.7%).

All our patients undergo ultrasound scanning of their kidneys by a urologist at each clinic visit to detect stones. Cystine stones are poorly radio-opaque and therefore X-ray is not performed routinely. Arguably, ultrasound may

underestimate the stone burden and CT would be the gold standard¹⁹. Repeated CT imaging however, would expose patients to excessive radiation and therefore not an appropriate tool for follow-up given the frequency to which many of these patients are seen in clinic (3 monthly to annually). Factors that may affect the diagnosis and interpretation of stones on ultrasound imaging include the presence of hydronephrosis, stone abutting renal sinus fat, the presence of bowel gas and body habitus¹⁹. The overall sensitivity and specificity of ultrasound for renal stones in the literature is 45% and 88% respectively⁸². From our clinical experience, we believe the sensitivity and specificity of the ultrasound performed by our specialist urologist is likely to be higher than that reported in literature. In our study, we have used our ultrasound findings as the gold standard comparison. Our results suggest that the use of urinary crystals may have comparable sensitivity and specificity to ultrasound imaging as reported in the literature. It would be interesting to compare the effectiveness of crystalluria measurement with cross-sectional imaging techniques, however, the numbers are likely to be small because of the avoidance of CT in this patient group.

Where possible, the urine samples were processed on the same day as the urinary pH of a sample may decrease the longer the sample is left out unprocessed, affecting the amount of crystals that precipitate⁸³. This may have practical implications if crystalluria is to become a point of care test for patients. Another study limitation is the small sample size, although this represents a relatively large cohort in a single institution for such a rare disease. Larger patient numbers can be obtained by collaboration with national and international groups such as

the UK Rare Renal Disease cystinuria Group, and the USA Rare Kidney Disease Consortium^{84,85}.

4.5 Conclusion

Our study suggests that EMU in cystinuria patients may be an oversaturated sample and may not accurately differentiate between patients with high and low disease activity. There is a higher correlation between presence of urinary crystals and presence of stones in CU samples. Furthermore, we found that the presence of urinary crystals in patients with cystinuria is associated with presence of stone formation and new stone growth when using the cytopsin method and clinic urine samples. The sensitivity and specificity of using crystalluria to determine the presence of stones or new stone growth is comparable to that of ultrasound. However more studies are required to fully characterize the use of crystalluria as a marker for disease activity, in particular comparing crystalluria to more sensitive cross sectional imaging. Whilst, we cannot currently recommend the use of crystalluria as a single diagnostic tool for identifying stone episodes, it may be a useful adjunct and requires further investigation.

5 The Genetic Diversity of Cystinuria in a UK Population

5.1 Introduction

Over 200 mutations have been described in Cystinuria but none have been reported in a UK population^{29,86-91}. We have over 80 patients coming through our clinic from all over the UK which allows us the unique opportunity to study one of the largest group of cystinuria patients. There have been few studies examining the genotype-phenotype correlation in cystinuria. In one study, 50% of patients with type A cystinuria present with stone disease in their first decade of life and another 25% in their teenage years³⁴. Another study comparing type A and B patients showed no difference in clinical or biochemical presentation between the two types of cystinuria¹⁰.

Given the number of different mutations described in Cystinuria, we devised a method of classification of patients with type A cystinuria into two groups for comparison. Group M included all patients with at least one missense mutation and Group N included all patients with a combination of all other types of mutations. We hypothesize that patients with missense mutations have less severe disease as missense mutations result in a change to a single amino acid within the protein compared with all other types of mutations (nonsense, frameshift, splice site, deletions and duplications) which cause larger genomic alterations. The levels of urinary dibasic amino acids and clinical parameters including age at presentation, number of stone episodes and number of interventions were compared between patients in groups M and N. We were unable to use this classification when looking at SLC7A9 mutations as all except

one patient had at least one missense mutation therefore precluding statistical analysis.

The aim of this study was threefold;

1. To identify the genetic mutations in a UK cohort of patients with cystinuria
2. To compare the urinary phenotype of type A patients with at least one missense mutation and type A patients with all other types of mutations.
3. To compare clinical phenotype (age of disease presentation, number of stone episodes and interventions) of type A patients with at least one missense mutation and type A patients with all other types of mutations.

5.2 Patients and Methods

All patients who attended the clinic between 1st August 2008 and 31st August 2012 were invited to participate in our genetic study. A single peripheral blood sample was taken for DNA sequencing and Multiplex Ligation-dependent Probe Amplification (MLPA). In addition, urine samples were taken at each clinic visit for measurement of spot urinary dibasic amino acids namely cystine, lysine, arginine and ornithine.

Ethical approval and adherence to best practice was obtained according to institutional guidelines.

5.2.1 DNA sequencing

DNA sequencing was performed using standard Sanger sequencing technique by RM⁹². Genomic DNA, extracted from the peripheral lymphocytes, was amplified by polymerase chain reaction (PCR) into 24 amplicons covering the whole exon and exon/intron boundaries of all coding exons of SLC3A1 and SLC7A9. A tag sequence was incorporated into the ends of each amplicon to facilitate the use of a universal primer in the sequence reaction of all amplicons. Products were detected on an ABI3730 DNA Analyser (Applied Biosystems) and analysed using Mutation Surveyor software (SoftGenetics). The clinical effect of each mutation detected was predicted using Alamut software (Interactive Biosoftware).

5.2.2 Multi-ligand Probe Amplification

MLPA was also performed to detect large deletions and duplications, that span one or more exon, which cannot be detected by DNA sequencing. However, the disadvantage is that point mutations can give false results and may look like a deletion as the probe doesn't bind. Two MLPA kits were used, one with all exons of the SLC3A1 as well as exon 13 of SLC7A9, and one kit with all coding exons of SLC7A9. Synthetic probes previously described⁹³ were used with control probes (on patients with known disease) from the P200 kits supplied by MRCHolland. 250ng of Genomic DNA and 50pM of each synthetic probe were used in the standard MLPA protocol, as described by MRC Holland for custom made MLPA assays using P200 control probes. Products were detected on an ABI3730 (Applied Biosystems) and analysed using GeneMarker software

(SoftGenetics). Samples were run with patients without cystinuria as well as samples without DNA. The analysis was also rerun for validity.

5.2.3 Genotype-phenotype Analysis

Data on patient demographics, ethnicity and age at first stone presentation and levels of urinary dibasic amino acids were recorded over the four-year period. In addition, data on the number of stone episodes and interventions including Extracorporeal Shockwave Lithotripsy (ESWL), Ureteroscopy (URS), Percutaneous Nephrolithotomy (PCNL) or open surgery where available was recorded for the four-year period.

Statistical analysis was performed using Graphpad software. The levels of urinary dibasic amino acids, age of disease presentation, number of stone episodes and interventions were compared between type A and B patients using Mann-Whitney U test. Results were reported as median and interquartile range. A p value of <0.05 was deemed as statistically significant.

In order to examine whether or not there was any correlation between a patient's genotype and clinical phenotype, we divided patients with SLC3A1 mutations into two groups. Group M included all patients with at least one missense mutation and Group N included all patients with a combination of all other types of mutations. Missense mutations result in a change to a single amino acid within the protein. All other mutations include nonsense, frameshift and splice site, and whole exon deletions and duplications. The levels of urinary dibasic amino acids

and clinical parameters including age at presentation, number of stone episodes and number of interventions were then compared between patients in group M and N using Mann-Whitney U test. Again, the results were reported as median and interquartile range. A p value of <0.05 was deemed as statistically significant.

5.3 Results

In total, 74 patients underwent genetic testing, 33 female 41 male. 85% (63/74) patients were white-British. Mean age at presentation to the clinic was 39.5 years (14-73). Mean age of presentation of disease was 23.9 years (0 – 61). The mean number of stone episodes per patient per year was 0.52 (range 0-3.75). The mean number of interventions per patient per year was 0.59 (range 0-2.75). There were five pair siblings⁹⁴.

41 patients had homozygous or compound heterozygous sequence variants in SLC3A1 including one where one of the sequence change was a variant of unknown clinical significance. 23 patients had homozygous or compound heterozygous sequence variants in SLC7A9 including 6 patients who had a variant of unknown significance. One patient had three sequence variants in SLC7A9 however two of which were of unknown significance. Three patients had a single heterozygous mutation in SLC7A9. Three patients had sequence variants in both genes (Type AAB), however the SLC7A9 variant was of unknown significance. No mutations were found in three patients.

There were 56 different pathogenic mutations identified. The commonest mutation in SLC3A1 was Ex5_9Dup 27% (24/88). The commonest mutation in SLC7A9 was c.614dupA (p.Asn206fs) 20% (11/55). p.Pro482Leu which affects more than 84% of the identified SLC7A9 alleles in the Japanese population is present in one patient who is half Japanese⁹⁰.

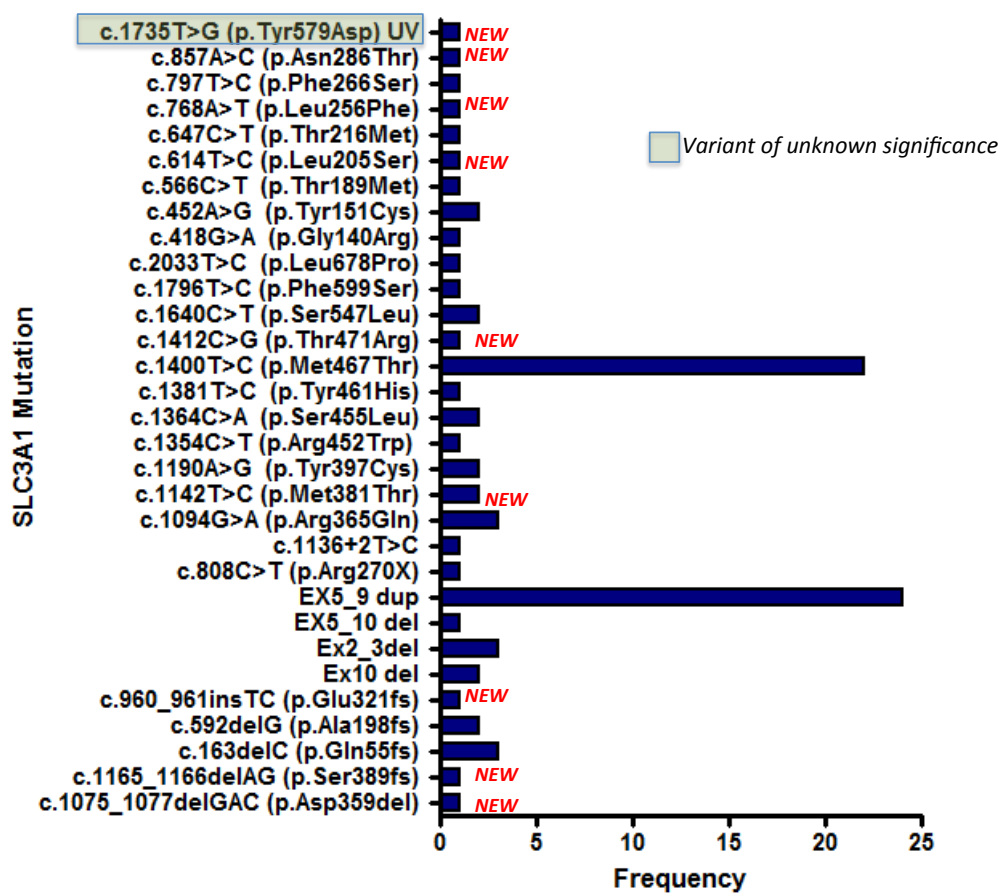


Figure 5.1 The different pathogenic mutations in SLC3A1 including 20 missense, 1 splice site, 5 frameshift, 1 nonsense and 4 deletions and duplications (31 in total including 9 new mutations).

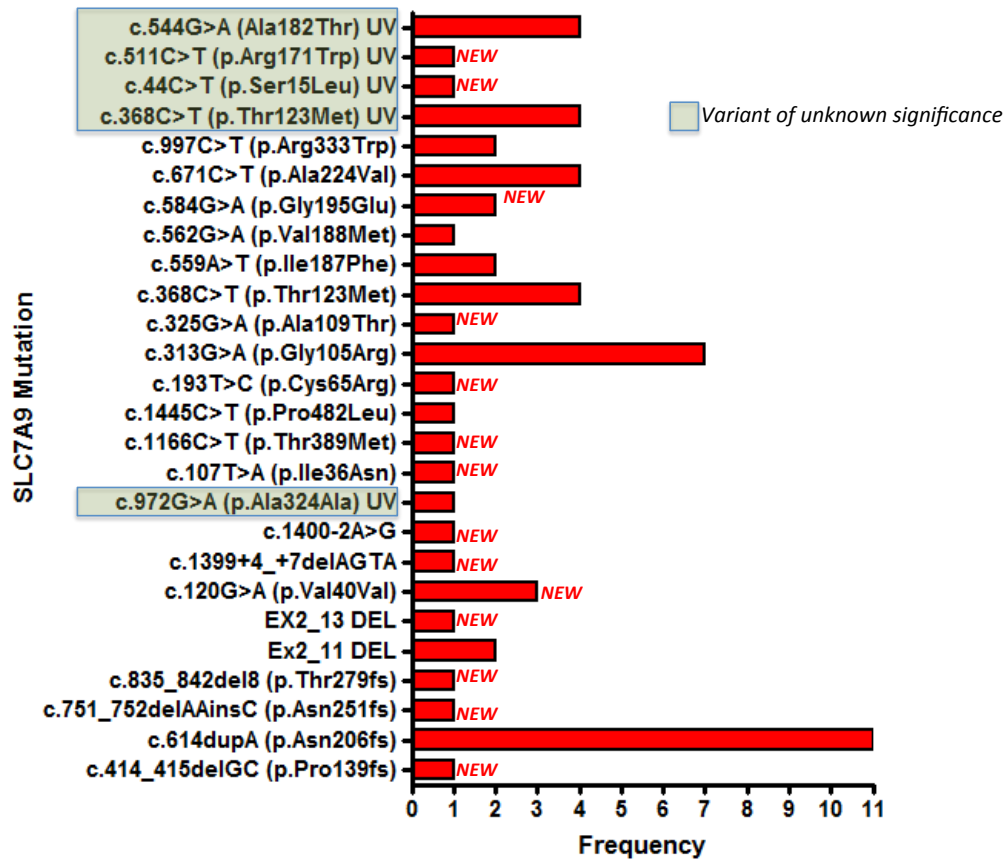


Figure 5.2 The different pathogenic mutations in SLC7A9 including 15 missense, 4 splice site, 4 frameshift and 2 deletions (25 in total including 14 new mutations).

23 previously unreported sequence variants were identified in our population of patients, three of these were of unknown clinical significance. There were three further variants of unknown significance which have been previously reported in literature.

There was no statistical difference in the levels of urinary dibasic amino acids between type A and B patients; cystine (177.7umol/mmol Cr IQR 139.3 to 221.8 vs 182.0umol/mmol Cr IQR 150.5 to 231.0 p=0.8327), lysine (613.0umol/mmol Cr IQR 549.0 to 729.6 vs 643.3umol/mmol Cr IQR 560.6 to 837.7 p=0.5307) arginine (226.9umol/mmol Cr 130.9 to 345.0 vs 294.3umol/mmol Cr IQR 76.19 to 391.2 p=0.4083) and ornithine (108.7umol/mmol Cr IQR 80.04 to 144.0 vs 111.0umol/mmol Cr IQR 79.19 to 159.6 p=0.8080). There was also no difference in age of presentation of disease (19.00 IQR 12.00 to 28.00 vs 16.00 IQR 14.00 to 35.00 p=0.7661), number of stone episodes per year (0.2500 IQR 0.0 to 0.5000 vs 0.2500 IQR 0.00 to 0.5000 p=0.7915) or number of interventions per year (0.5000 IQR 0.0 to 1.000. vs 0.2500 IQR 0.0 to 1.000 p=0.5569) between Type A and Type B patients with cystinuria. There were two SLC7A9 heterozygotes with a late onset of presentation of disease and low levels of dibasic amino acids. Type AB patients appeared to present with a later onset of disease (median 40) compared with Type A (median 19) and B patients (median 16) although this is based on three AB patients only.

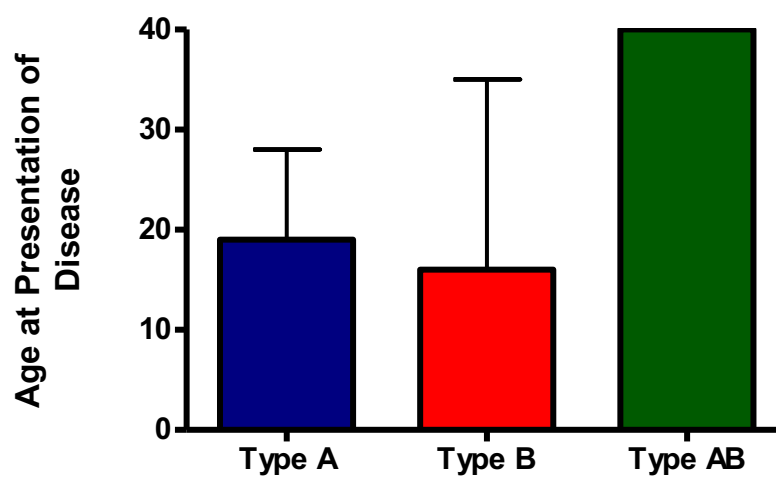


Figure 5.3 Comparison of age at presentation of disease between Type A, B and AB patients. There were only 3 type AB patients therefore it was not possible to perform statistical analysis on this group.

Patients with mutations in SLC3A1 were divided into group M and group N for comparison. There was no statistical difference between the levels of urinary cystine (169.8umol/mmol Cr IQR 138.8 to 214.7 vs 218.7umol/mmol Cr IQR 168.3 to 255.0 p=0.2343) in the two groups. There was however a statistical difference between the levels of urinary lysine (607.5umol/mmol Cr IQR 505.3 to 663.2 vs 735.1umol/mmol Cr IQR 676.1 to 857.0 p=0.0171), arginine (178.8umol/mmol Cr IQR 81.26 to 293.1 vs 386.1umol/mmol Cr 365.5 to 413.7 p=0.0008) and ornithine (98.76umol/mmol Cr IQR 79.24 to 135.9 vs 162.5umol/mmol Cr IQR 130.8 to 168.4 p=0.0211). This did not translate to a significant difference in age of presentation of disease (21 IQR 12.00 to 34.00 vs 16.00 IQR 14.50 to 19.50 p=0.1236), number of stone episodes per year (0.2500 IQR 0.0625 to 0.5000 vs 0.2500 IQR 0.0 to 1.000 p=1.000) or number of interventions per year (0.3750 IQR 0.0 to 1.000 vs 1.000 IQR 0.0 to 1.250 p=0.386).

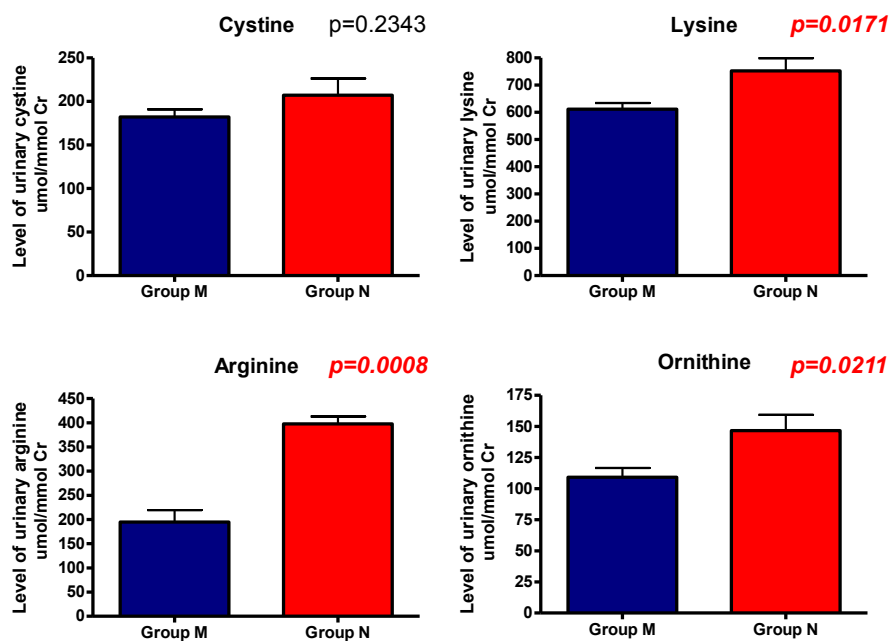


Figure 5.4 Comparison of levels of urinary dibasic amino acids between Group M and N.

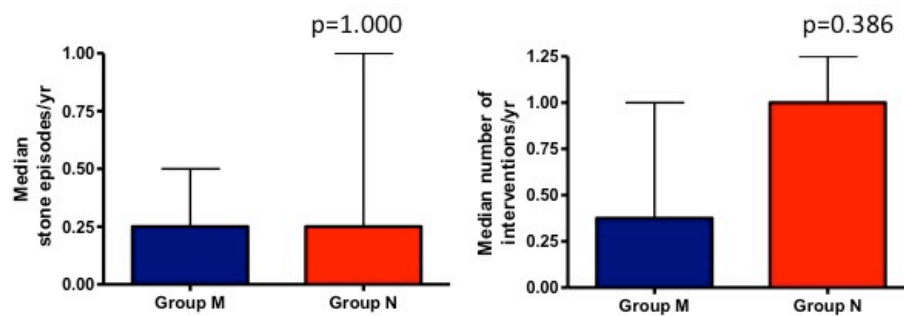


Figure 5.5 Comparison of stone episodes and interventions between Group M and N

5.4 Discussion

This is the largest single population study on genetic mutations in cystinuria in literature and the first in the UK. Figure 5.1 shows all the mutations found in both genes. Interestingly, we found 23 novel mutations not previously described in literature in our population. There were six variants of unknown clinical significance, three of which have not been previously reported. Variants were classified as being of unknown significance based on *in silico* predictions, frequency of the variant in studies reported in The Single Nucleotide Polymorphism Database (dbSNP), and presence or absence of other cystinuria variants detected. For example, one variant was found in a patient who had a well-described SLC3A1 mutation in the other allele. We believe the variant is likely to be pathogenic as no other mutation was found in this patient.

The majority of patients in this cohort were Caucasian (85%). The mutational diversity in our population is similar to pattern described by other European groups^{86,88,93,95-97}. Ex 5_9Dup was the commonest mutation in SLC3A1 in our population with a frequency of 27% (24/88) which has a worldwide frequency of 4.7%, 28.3% in Germany^{29,98}. Our commonest mutation in SLC7A9 was c.614dupA (20%), which has a worldwide frequency of 7% and 29% in Spain^{29,86,87,89}. The new mutations were found in both Caucasians and other ethnic groups.

Three patients had a single mutation in SLC7A9. These patients had slightly higher levels of all four urinary dibasic amino acids cystine (mean

92.65umol/mmol Cr), lysine (mean=306.13umol/mmol Cr), arginine (mean = 7.25umol/mmol Cr) and ornithine (mean=30.87umol/mmol Cr) than a normal population although in the lower range compared with other cystinuria patients. This is consistent with the clinical phenotype of obligate heterozygotes. There were six further patients who had a pathogenic mutation in SLC7A9 together with a variant of unknown significance. One patient had three sequence variants in SLC7A9 although two were of unknown significance. Both the variants of unknown significance have been seen in other patients with SLC7A9 pathogenic mutations.

Interestingly, there were three patients where no mutations were found in either genes. One patient had prior stone analysis confirming cystine stone formation. This patient had only marginally increased levels of all four urinary dibasic amino acids and relatively higher levels of arginine compared with the other three dibasic amino acids. A second stone sample sent for analysis showed calcium phosphate stone formation whilst on potassium citrate therapy.

The second patient where no mutations have been found with DNA sequencing and MLPA had pathologically high levels of all four urinary dibasic amino acids. Clinically, he has frequent stone episodes in keeping with a diagnosis of cystinuria. There are other such cases reported in literature. It is possible that DNA sequencing of exons may in fact potentially miss splice site mutations that are embedded in the introns. Mutations in genes not yet discovered may account for a handful of such cases ^{29,99}. More recently, Nagamori et al describes from studies done with co-immunoprecipitation and mass spectrometry a potential

second transporter protein AGT1 found on SLC7A13 which is involved in cystine transport and mutations of this gene may lead to disease¹⁰⁰.

The third patient underwent a ureteroscopy and laser fragmentation of a stone. During follow-up, he was found they had mildly elevated dibasic amino acids to normal range. Subsequent stone analysis revealed a calcium oxalate stone.

There were five pairs of siblings. Each pair shared the same mutations as anticipated. Despite having the same mutations, the clinical presentation in paired siblings was varied. This suggests that other epigenetic and environmental factors may play a role in the presentation of the disease.

There was no difference in the clinical presentation of patients between Type A and Type B cystinuria which is consistent with the other study in literature¹⁰. We had three Type AB patients with cystinuria who all had two SLC3A1 mutations and one SLC7A9 variant of unknown significance. All three patients presented with a later onset of disease which is again consistent with reports of other Type AB patients in published literature¹⁰.

Our prior studies demonstrate that urinary cystine is a poor marker of disease activity⁴. This is in part due to the supersaturation of cystine in the urine which precipitates into crystals. Cystine therefore exists in both soluble and insoluble form in urine and measurement of free urinary cystine only is not reflective of total levels. There are also limitations to current cystine assays as they do not distinguish between free cystine and cystine bound to thiol-binding drugs. This

may explain why, when comparing patients in group M versus patients in group N of the SLC3A1 gene, we found no statistical difference between the mean levels of cystine.

When looking at the other more soluble dibasic amino acids, there was a lower level of urinary lysine, arginine and ornithine in group M patients compared with group N patients. This difference was statistically significant for all three urinary dibasic amino acids. One possible explanation for this difference is that the presence of a missense mutation leads to a less deleterious effect on protein function. A missense mutation causes a change in a single amino acid whereas all other mutations (nonsense, frameshift, deletions, duplications and splice site) cause larger genomic shifts and are therefore assumed to have a greater effect on the protein. This explanation however, does not take into account the individual differences between all the different missense mutations. Some missense mutations will clearly have a greater effect on protein function than others. For example p.Gly105Arg is known to destroy the transporter activity and individuals with this mutation are likely to have severe disease¹⁰¹. By grouping all patients with missense mutations into one group, the individual differences between the different missense mutations are lost. This may in part account for the lack of clinical difference in stone episodes and number of interventions between patients.

Nevertheless, the results suggest for patients with SLC3A1 mutations, the presence of a missense mutation results in lower levels of urinary lysine, arginine and ornithine, potentially because the transporter protein is less severely affected

potentially due to compensatory effects of another transporter. Patients in group M also have lower levels of cystine than patients in group N, however, the lack of statistical significance may reflect the limitations of cystine measurement rather than a true lack of correlation. This is the first time such a genotype-phenotype correlation has been demonstrated in cystinuria. It was not possible to classify patients with SLC7A9 mutations in the same way because all these patients with the exception of one patient had at least one missense mutation. Furthermore, the inheritance pattern of SLC7A9 mutations is more complex than SLC3A1; whereas SLC3A1 is inherited in an autosomal recessive fashion and the less severely mutated allele is assumed to be expressed, SLC7A9 is autosomal dominant with incomplete penetrance. It is not possible to quantify the contribution of each mutated allele to the overall phenotype.

There was no difference in the age of presentation, number of stone episodes or interventions between group M and N patients. In addition to the limitations of our classification, there are several other explanations for the lack of clinical correlation. Firstly, we chose a four-year period over which we recorded stone episodes and interventions. This was because the start of this period represents the start of the clinic and data was collected prospectively. The medical records prior to this collected time period were often not available to us particularly when the patient was referred from other units. We found that patient's memories of stone episodes and interventions that occurred more than four years ago was unreliable particularly if they have had a number of procedures and if multiple procedures were for treatment of the same stone.

The number of interventions was also influenced by the frequency of clinic attendance. The more frequently we followed up patients, the more likely stones were detected and potentially more likely to intervene earlier than would otherwise have occurred if we had waited for patients to present with symptoms. Furthermore, all patients who attend our clinic undergo dietary intervention and some are on medical therapy to prevent further stone events. All these measures interfere with the natural progression of the disease.

In addition to understanding the genetic mutations in a UK population allowing for greater insight into the disease, our analysis suggests that for the first time, we can correlate a patient's genotype with biochemical phenotype; patients with missense mutations have lower levels of urinary lysine, arginine and ornithine. The correlation with cystine remains unclear and further improvements in cystine measurement are required to clarify this. This is the first step to a personalised approach to the management of patients with cystinuria. Finally, more research is still needed to understand what triggers the presentation of stone disease in these patients.

In this study, we did not examine the genotype or phenotype of family members. By doing so and adding our patients to the International Cystinuria Registry will facilitate further larger scale studies in collaboration with national and international groups such as the Rare Kidney Stones Consortium and the UK Rare Renal Disease cystinuria group (RaDaR) ^{84,85}. Further genetic studies are needed to elucidate how single mutations in the SLC7A9 gene can cause disease. With the recent discovery of SLC7A13 encoding for a second cystine

transporter, investigating mutations in this gene in patients where no mutations have been found might further explain these patients' phenotypes. Furthermore, the clinical value of the non-cystine dibasic amino acids is still unclear. In some patients we found that they had relatively higher levels of one dibasic amino acid over another.

5.5 Conclusion

The UK population of patients with cystinuria are mutationally diverse. We found 23 mutations not previously reported in literature. Patients with at least one missense mutation in SLC3A1 have lower levels of lysine, arginine and ornithine than those patients with all other types of mutations. Further research is required at a national and international level in collaboration with groups such as the Rare Kidney Stones Consortium and RaDaR.

6 The Role of Protein Modelling in predicting disease activity

6.1 Introduction

As previously described, patients with cystinuria are clinically and genetically diverse. We have found 57 different mutations in our UK cohort of which 23 have not previously been described⁹⁴. Frame shift, deletion, duplication, splice site and nonsense mutations typically result in large effects on the encoded protein and therefore its protein structure or function. Most mutations described in Cystinuria however, are missense mutations. A missense mutation is a single point mutation, which results in a change to a single amino acid in a protein. Some missense mutations can render a protein non-functional whilst others may not lead to any appreciable protein change. For many of the missense mutation in SLC3A1 or SLC7A9, it is not clear what effect they have on protein function and how they lead to disease presentation.

The aim of this study was to use protein modelling to model the rBAT and b(0+)AT subunits and identify how the missense mutations identified in our cohort may affect protein function. Similar protein modelling approaches applied to other conditions such as chronic kidney disease have been successfully used to identify candidate genetic variants identified in genome wide association studies^{102,103}. Understanding how individual genetic mutations can cause protein dysfunction could lead us to a greater insight to the range of clinical phenotypes of cystinuria and potentially allow us to predict a patient's clinical severity based on their genotype. This is the first step towards a personalised tailored approach to management of patients with cystinuria.

6.1.1 The rBAT and b(0+)AT protein unit

SLC3A1 encodes for the neutral and basic amino acid transport protein (rBAT) and SLC7A9 encodes for the light chain b amino acid transporter b(0+)AT^{13,14}. The proteins form a dimer linked by a disulphide bridge¹⁵. b(0+)AT consists of 487 amino acids (53481 Da) with 12 transmembrane helices that form the channel through which dibasic amino acids (cystine, lysine, arginine and ornithine) are transported into the cell with the exchange of neutral amino acids. rBAT is the larger of the two subunits consisting of 685 amino acids (78852 Da), with a single transmembrane helix and a large extracellular domain. Experimental studies suggest that rBAT may function as a mediator of b(0+)AT^{14,16}. Mutations in either of these two genes can result in defective transport of dibasic amino acids across the renal tubular membrane and intestine^{104,105}.

6.2 Patients and Methods

I was responsible for designing this study, collecting the clinical data, and performing the data analysis. Dr Mark Wass performed the protein modelling and scoring of the mutations. Dr Mark Wass and I contributed equally to the final paper.

6.2.1 Protein Modelling

The protein structures of rBAT and b(0+)AT were modelled using the Phyre2 web server¹⁰⁶ (Figure 2). The rate4site algorithm from ConSurf¹⁰⁷ was used to

calculate evolutionary conservation of the residues in both genes. Functional sites of the protein were modelled using multiple methods. The ligand binding sites including the amino acid, sugar and calcium binding sites were modelled using 3DLigandSite^{108,109} and *Firestar*¹¹⁰. *Pfam* was used for predicting the active site residues in SLC3A1¹¹¹. The VarMod webserver was also used to investigate the likely functional effects of the mutations¹¹²

The b(0+)AT protein transports dibasic amino acids into the cell in exchange for neutral amino acids. There are therefore two sites for amino acid binding, one on each side of the transporter. The luminal facing binding site was modelled using 3DLigandSite and *Firestar* using the Arginine bound to another related APC transport (AdiC, pdb code: 3OBM)¹¹³. To model the intracellular facing conformation, the putative binding site identified for ApcT (another member of the APC transporter family) was mapped onto our model¹¹⁴.

Prior studies have proposed that Lys158 in ApcT has a role equivalent to sodium in sodium dependent transporters¹¹⁴. This lysine is conserved in b(0+)AT (Lys 184) and 3 of the 4 residues coordinating with it are also conserved (G41, I44, S312). The residues from the three different sites in b(0+)AT were used as indicators of functionally important regions where mutations are likely to affect the function of the transporter.

6.2.2 Mutation Analysis

A number of mutations have been previously described. Where prior experimental studies were available, this was used to predict the effect of the mutation in our protein model. For mutations where no prior experimental data was available, we used our model to predict how each individual mutation can lead to a change in protein conformation.

6.2.3 Genotype-phenotype analysis

The mutations in SLC7A9 were then scored by a non-clinical investigator (MW) blind to the clinical data into those predicted to cause a low/low-medium effect=1 or high/high-medium effect=2 on protein function. Each patient with homozygous/compound heterozygous mutations in SLC7A9 has two mutations and therefore were assigned an overall severity score based on the sum of the score of each individual mutation. The levels of urinary dibasic amino acids, clinical parameters i.e. age of presentation of disease, stone episodes and interventions as recorded over a four-year period were then compared between patients with a high and low score using the Mann-U Whitney test. Patients who had mutations that were not modelled were excluded from the analysis.

6.3 Results

A total of 74 patients were genotyped for mutations. There were 41 patients with mutations in SLC3A1 including one patient with a variant of unknown

significance. There were 23 patients with compound heterozygous mutations in SLC7A9 including 6 patients with variants of unknown significance. Three heterozygous patients had a mutation in only one allele in SLC7A9. One patient had three SLC7A9 mutations although two were unknown variants. Three patients had mutations in both genes. We have not found mutations in either genes in three patients.

There were 55 different pathogenic mutations in total. There were 20 different missense mutations in SLC3A1 and 15 different missense mutations in SLC7A9.

6.3.1 The rBAT protein (SLC3A1 mutations)

The residues predicted to have a potential functional role in the alpha amylase domain for sugar binding are: D172, Y175, H215, V258, Y259, F278, M279, Q282, S312, D314, A315, F318, E384, D449. Additionally D133, N135, D137, N139, D141 are predicted to bind calcium.

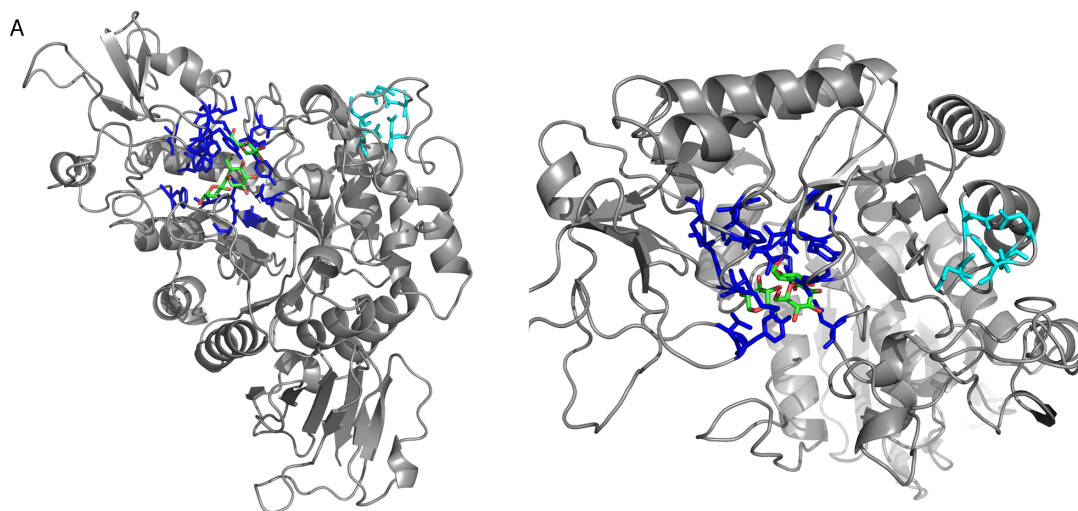


Figure 6.1 Model of rBAT. The modelled sugar binding site residues are blue and the calcium binding is light blue

Interestingly, for the majority of the rBAT mutations observed, the structural analysis suggests that the mutations may have an effect on the structure or stability of the protein. These mutations fall into two main groups. In the first group, a hydrophobic amino acid is replaced by a polar or charged amino acid (p.Tyr151Cys, p.Leu205Ser, p.Tyr397Cys, p.Tyr461His, p.Met467Thr, p.Tyr579Asp, p.Phe599Ser) where the hydrophobic side chain is typically buried and packed against other hydrophobic side chains (Figure 3). In the second group, a polar or charged amino acid is replaced by a hydrophobic side chain (p.Thr189Met, p.Thr216Met, p.Arg452Trp, p.Ser547Leu) and modelling suggests that these mutations remove polar contacts that stabilise the protein structure. These four positions are highly conserved in homologous proteins indicating an important structural or functional role. Two mutations (p.Leu256Phe, p.Thr471Arg) that may affect the stability/packing of the protein do not fit into these two groups. For p.Leu256Phe, the increase in size of the side chain is likely to alter the side chain packing in this area and for p.Thr471Arg there is an increase in both charge and size and this side chain is packed against an adjacent beta sheet.

Four other mutations are located close to “functional” regions of the protein. p.Gly140Arg is present within the loop where calcium is modelled to be bound (Figure 3). This position is completely invariant in homologues suggesting an important structural/functional role for this residue. Introduction of a positively charged arginine may be expected to interfere with the binding of the positively charged calcium ion. p.Thr189Met is located in the alpha helix adjacent to the calcium binding site so it is possible that destabilisation here could affect the

calcium binding site (Figure 3). Two of the mutations (p.Met381Thr, p.Tyr397Cys) are close to what would be the active site if the protein was an active hydrolase. p.Met381 is highly conserved in orthologues and the mutation to threonine could introduce a polar contact with p.Asp369. For p.Tyr397Cys the mutation is likely to remove a polar contact.

6.3.2 The *b(0+)*AT protein (SLC7A9 mutations)

The functional residues identified in SLC7A9 for amino acid binding are: I38, I43, S46, G47, W230, A231, Y232, T91, K92, E96, L117, K121, S124, I128, I371, L374, V375, F378. Other functional residues are G41, I44, K184 and S312, which are likely to be important for transport. As the mechanism of transport is not clearly understood there are likely to be further residues that are functionally important that have not been identified here.

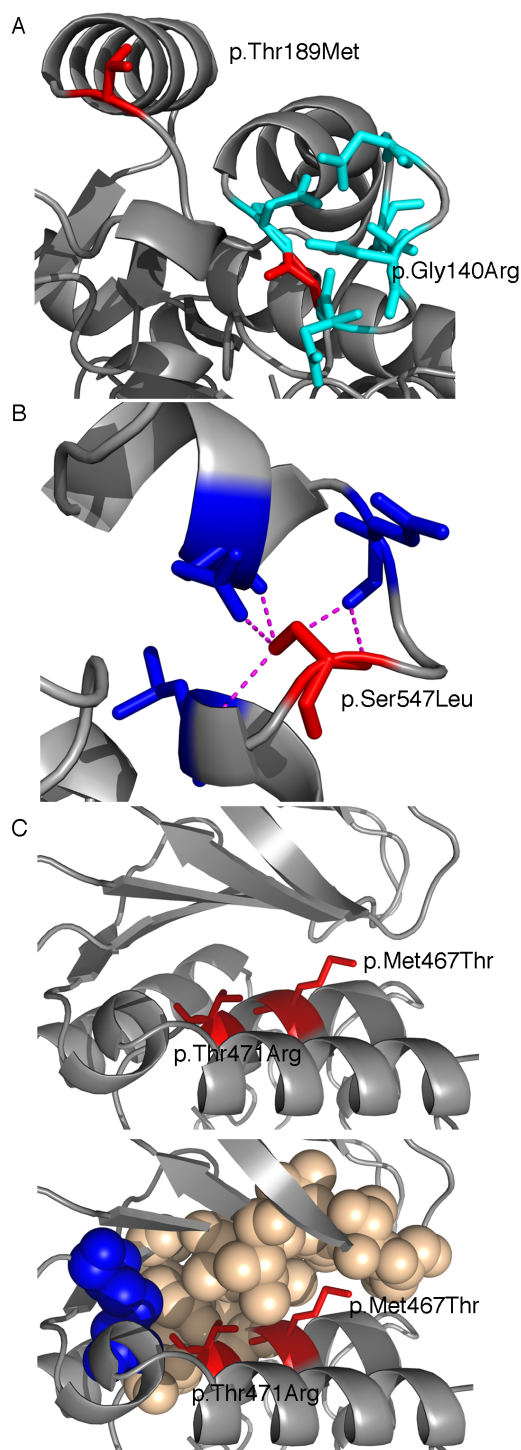


Figure 6.2 Example mutations in rBAT. In all figures the residues that are mutated are red in stick format with the wild type side chain. A) mutations close to predicted functional residues that bind calcium (Calcium binding residues are light blue). B) A mutation altering polar contacts, p.Ser547 makes polar contacts with multiple residues (dark blue), polar contacts indicated in purple. Mutation to leucine would remove these polar contacts. C) Mutations that introduce polar or charge side chains in hydrophobic regions. In the bottom image hydrophobic residues that contact the residues that are mutated are shown as beige spheres. p.Arg584 an arginine close to p.Thr471 is coloured blue.

Finally, the structural model does not include p.Leu678Pro; this position is highly variable in homologues and there is little information to suggest how the mutation may alter the protein structure or function.

B

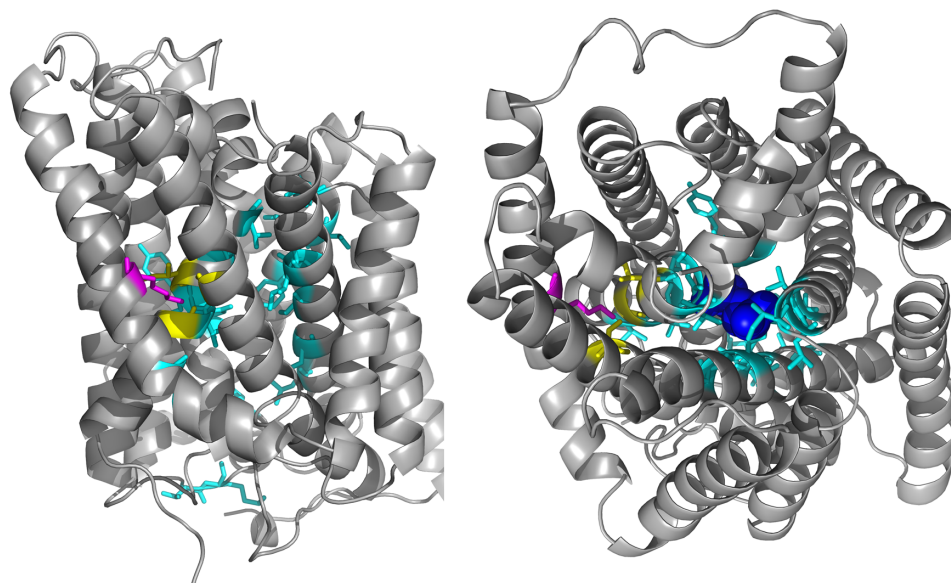


Figure 6.3 Model of b(0+)AT, Residues modelled to contact the transported amino acids are light blue. The conserved p.Lys184 is purple and the conserved residues coordinating with it yellow

Some of the 15 different missense mutations present in SLC7A9 have been previously identified and for some of them experimental studies have suggested how they alter protein function¹⁰¹. Overall, we found that mutations in SLC7A9 are primarily either close to functional binding sites or close to the end of transmembrane helices.

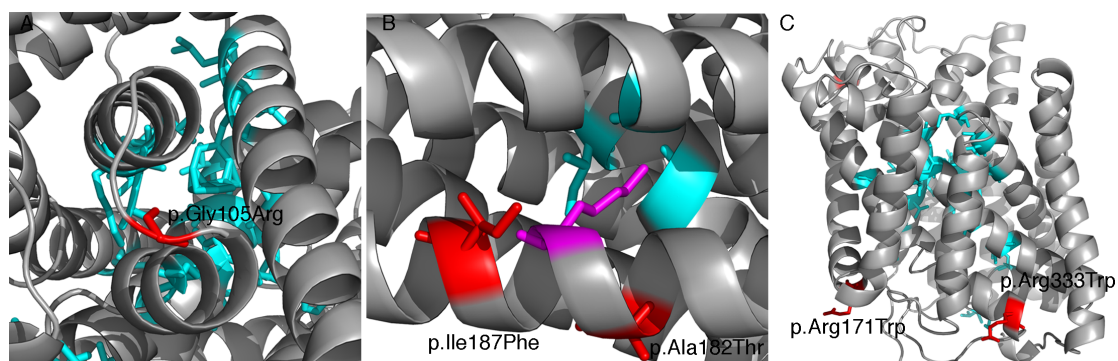


Figure 6.4 Example mutations in b(0+)AT. In all images the mutated residues are displayed as red sticks in their wild type format. Predicted functional residues are coloured light blue. A). The mutation p.Gly105Arg would introduce a larger and charged side chain that is close that may reduce accessibility for amino acids to enter the transporter. B). p.Ile187Phe and p.Ala182Thr mutations are adjacent to p.Lys184 which is thought to play a role equivalent to sodium in sodium dependent transporters. C). Mutations close to the end of transmembrane helices may reduce stability in the membrane.

6.3.3 Genotype-phenotype analysis

The commonest missense mutation in SLC3A1 is p.Met467Thr with a frequency of 25% (22/88). The most common missense mutation in SLC7A9 is p.Gly105Arg with a frequency of 13% (7/55).

The levels of dibasic amino acids associated with each mutation in SLC7A9 are seen below.

Figure 5.

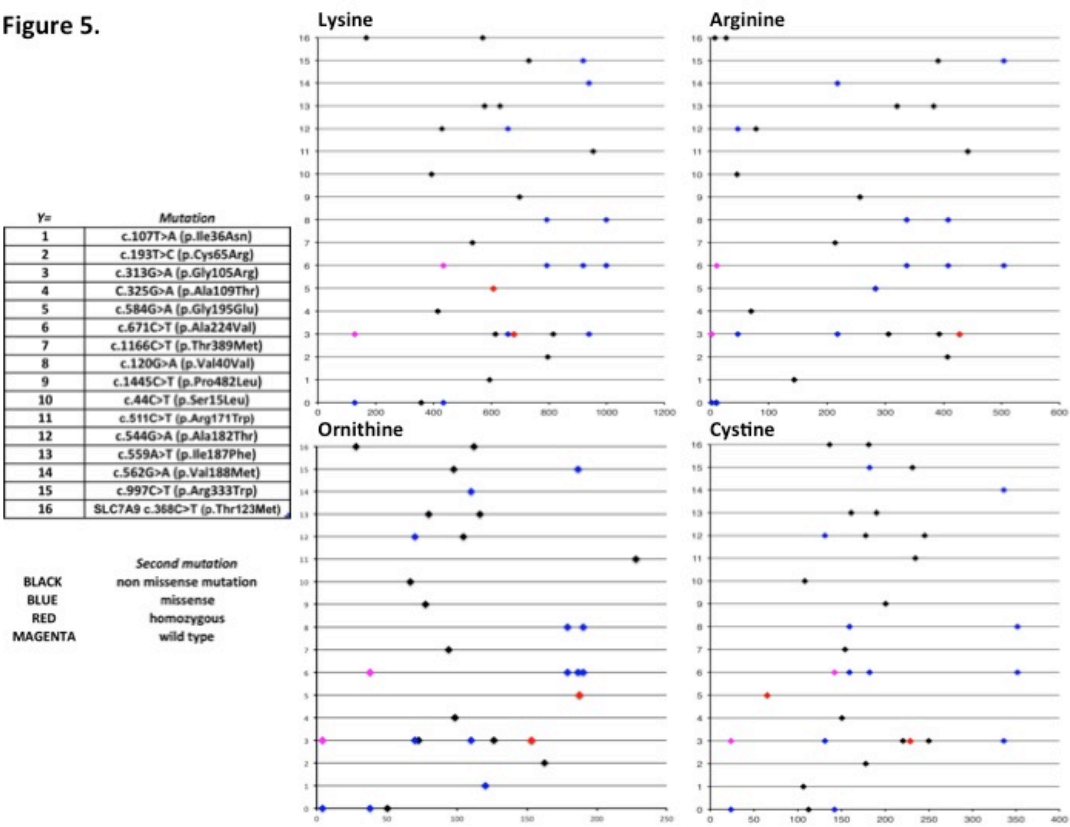


Figure 6.5 The urinary phenotype for each mutation in SLC7A9. The x-axis refers to the levels of dibasic amino acids (umol/mmol Cr). The y-axis represents the first mutation. The dot represents the second mutation. The second mutation is colour coded based on whether it is non-missense, missense, homozygous or wild type.

The range of urinary phenotype for each mutation is varied; compound heterozygous patients who share one mutation can present with a range of urinary phenotypes. From our severity scoring classification in SLC7A9, 11 patients scored 4 and nine patients scored 3. Two heterozygous patients with only one mutation scored 2, and one heterozygous patient scored 1. When comparing the urinary dibasic amino acid levels between compound heterozygous patients with a higher severity score (S=4) versus a lower score (S=3), patients with a higher score had higher levels of dibasic amino acids. This was statistically significant for arginine (383 umol/mmol Cr IQR 283 to 392.7 vs 70 umol/mmol Cr IQR 36.38 to 237.3, $p=0.0151$) and ornithine (120.2 umol/mmol Cr IQR 97.67 to 153.1 vs 94 umol/mmol Cr IQR 68.38 to 111, $p=0.0482$), but not cystine (201 umol/mmol Cr IQR 161 to 231 vs 154 umol/mmol Cr IQR 133.7 to 191.3, $p=0.2545$) or lysine (629.5 umol/mmol Cr IQR 593.2 to 814.6 vs 569.5 umol/mmol Cr IQR 403.6 to 807.5, $p=0.2887$)¹¹⁵.

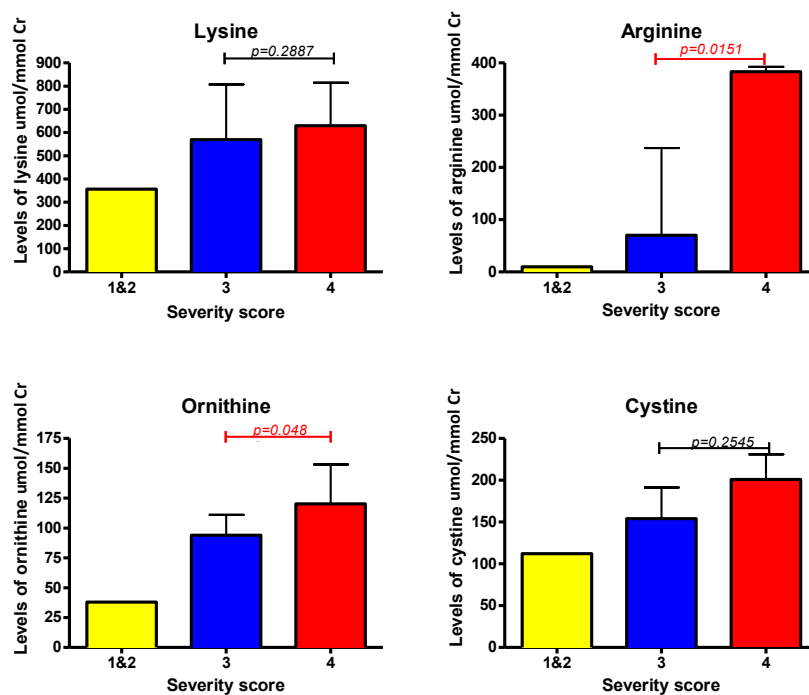


Figure 6.6 Comparison of urinary phenotype of patients with a severity score of 3 and 4. Group 1&2 (yellow) represent heterozygotes with only one mutation. There were only 3 patients in this group therefore statistical analysis was not possible.

Patients with a higher severity score had a higher number of stone episodes (0.5000 IQR 0 to 1.000 vs 0.0 IQR 0 to 0.2500, $p=0.0451$) and showed a tendency towards a younger age of disease presentation (15.5 IQR 14 to 32.5 vs 29 IQR 14 to 40.5, $p=0.2081$), and more interventions per year (0.6250 IQR 0 to 1.125 vs 0.1250 IQR 0 to 0.5000, $p=0.1569$) although these differences was not statistically significant.

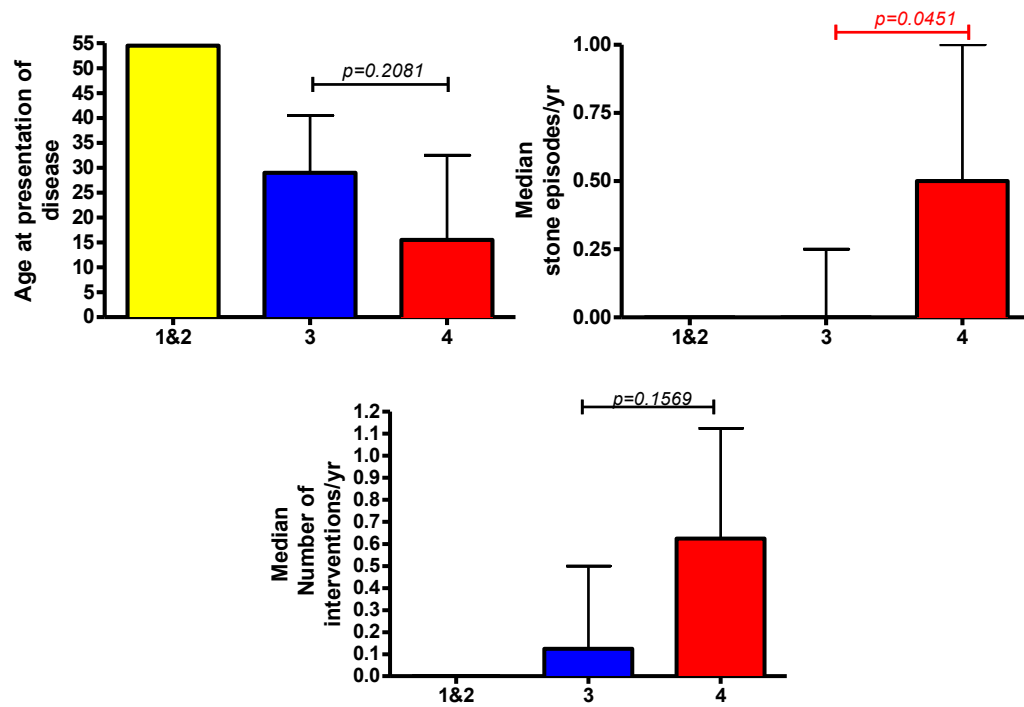


Figure 6.7 Comparison of clinical phenotype of patients with a severity score of 3 and 4. There were only 3 patients in group 1&2 therefore statistical analysis was not possible.

The three heterozygous patients with only a single mutation (S=2) had lower levels of all four dibasic amino acids (*cystine* 112.3 IQR 23.67 to 142, *lysine* 356.7 IQR 127.7 to 434, *arginine* 9.833 IQR 2.167 to 11 and *ornithine* 38 IQR 4.167 to 50.43) than all compound heterozygotes.

6.4 Discussion

Genetic testing revealed a total of 56 different pathogenic mutations in our UK cohort, the majority of which are missense mutations. Whilst frameshift, nonsense, splice site, deletions and duplications cause large genomic alterations and therefore lead to protein dysfunction, it is unclear how missense mutations may affect protein structure and function and play a role in disease.

Using structural models to investigate SLC3A1 mutations is more complicated than for SLC7A9 mutations because the function of rBAT is not clearly understood. Interestingly in rBAT, few mutations directly affected the predicted functional residues that would be associated with the enzyme activity that is typically present in this family of proteins. However, a large number of mutations introduce a charge into a buried region or hydrophobic region and could therefore disrupt protein folding or reduce stability. This is consistent with what we know of the rBAT protein; experimental studies suggest that the heavy rBAT subunit is essential for cell surface expression of b(0+)-AT¹⁴ and essential for transport of the heterodimer to the plasma membrane. There is evidence to suggest that the extracellular glycosidase domain has a role in cystine transport but not the other dibasic amino acids¹¹⁶. rBAT also requires

chaperones to fold correctly and a number of mutations have been linked with incorrect folding of the protein and/or trafficking to the plasma membrane ¹¹⁷. The interaction between proteins *in vivo* cannot with existing protein models be fully appreciated.

rBAT contains an alpha-amylase domain. These domains typically function as enzymes that breakdown large linked polysaccharides (e.g. starch) into simple sugars such as (glucose). It is not clear if the alpha amylase domain in rBAT performs this function and how this may relate to the function of amino acid transport. The presence of few mutations close to the functional region of this domain agree with observations that this domain is not functionally active.

It is known from prior experimental studies that the light chain b(0+)AT encoded by SLC7A9 forms the exchanger of dibasic amino acids for neutral amino acids^{104,105,118,119}. It has also been suggested that the light subunit may be fully functional even in the absence of the heavy subunit in transfected HeLa cells¹²⁰. There appear to be two main ways in which mutations may affect this light chain subunit protein. Multiple mutations are observed close to functional sites in the protein and it appears that even conservative mutations (i.e. change to amino acids with similar properties) in these regions are sufficient to alter b(0+)AT function. A number of other mutations possibly alter the stability of the protein in the membrane.

Whilst many mutations are present in the study, only a few are present in multiple individuals. For SLC3A1, only two of the missense mutations are present in two or

more patients (p.Ser547Leu and p.Met467Thr). Given this limited data, it is not possible to draw conclusions on any individual mutation alone.

We observed from our prior study that in patients with mutations in SLC3A1, the presence of at least one missense mutation corresponded with patients having significantly lower levels of lysine, arginine and ornithine but not cystine than patients with all other types of mutations⁹⁴. Our data suggests that the presence of a missense mutation is likely to result in a less severe phenotype than all other combinations of mutations.

An alternative approach was adopted for SLC7A9 using the structural model and existing experimental data to assess the level of effect each mutation is likely to have on protein function. Each mutation was assigned a score of 1 = low/low-medium effect and 2= high/high-medium effect. All non-missense mutations were assigned a score of 2 in keeping with our assumptions that these mutations cause large genomic rearrangements and consequently protein dysfunction. The missense mutations were scored from our modelling predictions. The score was determined by an investigator blinded to the clinical data and based on several factors including the proximity of the mutation to the predicted functional sites and size of conformational change. Where patients had two mutations, the overall severity score was calculated as the sum of the score of each individual mutation.

As there were only three heterozygous patients with a severity score of 2, they were omitted from the analysis. All three heterozygous patients had lower levels of dibasic

amino acids than compound heterozygotes in keeping with other studies¹⁰. Interestingly, in compound heterozygotes, patients with a higher severity score (S=4) had higher levels of urinary dibasic amino acids compared to patients with a lower severity score (S=3). This was statistically significant for arginine and ornithine but not for cystine and lysine. This finding suggests that protein modelling may help predict a patient's urinary phenotype. The lack of statistical significance for cystine and lysine may reflect the limitations of cystine measurement⁴ and the unique transport mechanisms for lysine⁷⁵, compounded by a small sample population. However, we recognise that the findings may also simply indicate a lack of statistical association and more work is needed to explore our hypothesis. Patients with a higher severity score had more stone episodes per year and showed a tendency to present earlier with disease and had more interventions per year, although the difference was not statistically significant for the latter two.

One of the limitations of performing any research in cystinuria is the lack of validated measures of disease severity. Any clinical intervention including dietary or medical therapy can potentially affect the natural progression of the disease. Collaborations with other National and International groups can help to establish a standardised measurement of clinical severity to enable further research on genotype-phenotype associations^{84,85}. Clearly, a patient's genotype can only determine part of the patient's disease profile and clinical phenotype may also be influenced by modifier genes, anatomical factors and complex genetic and environmental interactions. An equivalent analysis of SLC3A1 was attempted but given the lack of understanding of

the function of rBAT, it was not possible to confidently assign many of the mutations to a score.

The protein model is generated using a related protein as a template. i.e. the two protein sequences are related and one of them has a solved structure. In this case, the sequence encoded by p.Leu678Pro is not present in the template protein and therefore not modelled. This is the same for p.Ser15Leu and p.Pro482Leu, present at the N and C termini respectively in b(0+)AT. The model has also not offered any suggestions as to how some mutations can affect protein function. p.Val40Val is a synonymous single point mutation resulting in the translation of the same amino acid (i.e. val to val). Our protein model does not suggest how silent mutations affects protein function although splicing prediction programs suggest that p.Val40Val affects splicing. This phenomenon is noted in studies of other diseases also ¹²¹. Patients with these mutations have not been included in the analysis and their true effect on protein function should ideally be experimentally verified.

The advantage of protein modelling is that obtaining experimental data can be time consuming, particularly given the number of different mutations in Cystinuria. Prospective evaluation is required to assess whether it is possible that the classification we have proposed can suggest an indication of severity of an individual's disease.

6.5 Conclusion

Protein modelling has given us insight into how the different missense mutations may cause the range of phenotypes seen in cystinuria. Patients with a high severity score as predicted by our protein model of b(0+)AT appear to have higher levels of urinary arginine and ornithine and more stone episodes than those with a lower score. This is a step closer to a personalised approach to the management of patients with cystinuria.

7 Conclusion

7.1 Summary of Results

There is a wide variation in the phenotype of patients with Cystinuria, this can make the management of this rare disease difficult to predict and monitor. My research was aimed at exploring potential clinical markers of disease activity and understanding the genetic mutations that cause this disease.

7.1.1 The Role of Urinary dibasic amino acids

Cystinuria results in an accumulation of urinary cystine and the urinary dibasic amino acids lysine, arginine and ornithine. The levels of urinary cystine have not been shown to accurately reflect stone disease or disease severity. Several studies have described the inherent limitations of cystine measurement⁶⁴. We evaluated the utility of the other urinary dibasic amino acids in predicting stone formation. We found there were higher levels of urinary cystine, lysine and arginine in stone formers compared to non-stone formers although the differences were not statistically significant. Only urinary ornithine appeared to be associated with stone formation ($p < 0.05$). If urinary ornithine can predict whether a patient is likely to form stones, this may be useful in the initial assessment of a patient with cystinuria. However, we recognise the limitations in its use for monitoring disease activity as most drugs used in the management of this disease aim to affect the levels of urinary cystine and do not as far as we know have any effect on the other dibasic amino acids. The use of the other dibasic amino acid however may be useful in the future if protein targeted therapies

are developed. This was the first time that the prognostic value of the non-cystine urinary dibasic amino acids has been investigated.

7.1.2 The Role of Urinary crystals

Our next step was to look at the association between the presence of urinary crystals and stone formation. We compared EMU and CU samples in patients with stone disease and new stone formation and two methods of counting crystals. We found a better association between CU and stone disease compared with EMU. Furthermore, we found that CU samples analysed by conventional cytopsin had the highest AUC compared to CU samples analysed by Malassez and EMU samples analysed by both methods. This is because large aggregates of crystals are often underestimated by the Malassez counting chamber method. In this study, we were not able to replicate the solubilisation of cystine crystals following the method described by Dolin et al.

Whilst crystalluria alone is not robust enough as a single diagnostic tests, we propose that crystalluria may be useful when used in conjunction with other tests i.e. urinary dibasic amino acids, much like the use of PSA in a prostate cancer nomogram.

7.1.3 Genetics of Cystinuria in a UK population

No genotype-phenotype correlation has previously been demonstrated in literature when comparisons of patients with mutations in SLC3A1 and SLC7A9 have been made¹⁰.

This was the first study of a UK cystinuria population and we found our cohort to be genetically diverse with 57 mutations of which 23 were not previously described. The majority of mutations in our cohort were missense mutations. We compared patients with at least one missense mutation in SLC3A1 with patients with combinations of all other mutations. We hypothesized that missense mutations would cause less protein dysfunction than all other mutations. We found that patients with at least one missense mutation had lower levels of the urinary dibasic amino acids. This was statistically significant for urinary lysine, arginine and ornithine but not cystine. This demonstrated for the first time that a patient's genotype can determine urinary phenotype. The lack of a statistically significant relationship for urinary cystine may be due to the limitations in cystine measurement as previously described⁶⁴.

We examined the correlation between genotype and clinical phenotype using our classification. Although we found that patients who had at least one missense mutation were less likely to form stones and require fewer interventions, this association was not statistically significant. There were however some recognised limitations to how the data was collected and recorded that may account for this. For our study, we defined a stone episode as any episode of stone that has been reported

by a patient including those passed spontaneously, those causing colic and stones that require intervention over a four-year period. We found that patient recall of their stone episodes was often unreliable. Furthermore, one can argue that this method of reporting is too simplistic as not all stones are the same. Stone burden can vary and a stone requiring PCNL may arguably have greater ‘weight’ than one that can be treated ureteroscopically. Likewise, a stone requiring intervention has greater ‘weight’ than a stone that can pass spontaneously. Therefore, it is fundamental that in order for further research into this disease to continue, a more standard instrument for evaluating clinical disease severity must be established.

Furthermore, some patients were followed up more frequently than others. The factors that influence this included the patient’s initial presentation and stone burden, frequency of stone episodes, whether they were on any medications that required monitoring, and renal function. Cystinuria affects predominantly a young working population and clinicians must balance the frequency of clinic visits to the disruption to patient’s lives. These were all considerations we recognise as limitations to conducting research in an opportunistic environment and may account for the lack of statistical association in some of our results.

It was not possible to apply this genetic classification to evaluate patients with SLC7A9 mutations. Firstly, all patients with SLC7A9 mutations except one had at least one missense mutation therefore making statistical comparisons impossible. Secondly, the inheritance pattern for patients with SLC7A9 is less well defined; patients can have one mutated allele and present with cystine stones.

The results of this study on one of the largest population of patients with cystinuria show for the first time a genotype-phenotype association that is applicable to patients with mutations in SLC3A1. Patients with at least missense mutations have a biochemically less severe phenotype as evidenced by lower levels of urinary lysine, arginine and ornithine. This potentially has a role at initial diagnosis at determining frequency of follow up.

7.1.4 The Role of Protein Modelling

Due to the limitations of our classification described above, a different approach was adopted for examining SLC7A9 mutations. We applied a validated protein modelling software to determine the effect mutations would have on the end-protein. We found we were able to determine the location of mutations in relation to the ligand-binding sites and the degree of conformational change. We used this model to classify our mutations in SLC7A9 into those that were predicted to cause a high effect (S=2) and those predicted to cause a low effect (S=1). Patients with heterozygous mutations were then given an overall score; a sum of the individual scores of each mutated allele.

We found that patients with a score of 4 had higher levels of urinary arginine and ornithine than patients with a score of 3. Patients with a higher score also had more stone episodes.

This finding demonstrated that protein modelling may partly help us to understand the genetic mutations identified in our UK cohort. It is uncertain the role of protein modelling in examining mutations in SLC3A1 as the role of the rBAT protein is less clearly understood. Experimental studies are time-consuming and the results would lag significantly behind the rate at which new mutations are being reported. Using protein modelling, we can suggest how individual mutations can lead to protein dysfunction. This is the first step towards a personalised approach to the management of patients with this disease.

7.2 Implications for Current Practice

Patients with cystinuria are clinically and genetically diverse. Given the wide range of presentations, patients need a tailored approach to the management of their disease. Currently, a method of determining a patient's disease severity is lacking, and only following a period of observation can we determine whether someone has 'mild' or 'severe' disease.

There are currently no clinical markers to help us monitor patients with cystinuria. Measurement of urinary cystine is flawed as it exists in both soluble and insoluble form. Our study findings demonstrate that urinary ornithine may have some use in the initial assessment of a patient but currently lacks value in the monitoring of patients particularly if they are on thiol-binding medication.

Prior studies suggested that early morning urine is the most accurate assessment of urinary cystine due to the overnight saturation of the cystine. However, we have found that clinic urine is better associated than early morning urine for stone disease. Again, clinic urine is strongly associated with stone disease but on its own, it is not robust enough to be of diagnostic or prognostic use. It may be possible to use this association in conjunction with other tests as a marker for predicting a patient's disease severity.

With regards to genetics, for the first time we can use a patient's genotype to make suggestions on urinary and clinical phenotype. We may be able to use this information to tailor a patient's management and determine the frequency of clinic visit. Clearly, a patient's genotype can only determine one aspect of a patient's disease and there are currently unknown epigenetic and environmental factors that would influence a patient's clinical course. By understanding how individual mutations can affect protein function, this has led to greater insight into this disease and provide a model whereby protein targeted therapies can be tested.

7.3 Future Challenges

My research has provided a better understanding on the utility of the cystine and the other dibasic amino acids in predicting severity of cystinuria. Urinary ornithine appears to be associated with stone formation and can act as a proxy for disease severity. However, its usefulness is hampered as current treatments in cystinuria aims

to reduce cystine levels only and as far as we know, does not affect ornithine levels. We have also found that the levels of urinary dibasic amino acids are increased to varying degrees. We do not currently understand why some patients have higher levels of one dibasic amino acid compared to another and further research into this may be useful. For example, further understanding of why individual urinary dibasic amino acids levels vary may help to develop a therapy for the selective transport of urinary lysine, arginine and ornithine in place of cystine.

We observed whilst doing this work using ion-exchange HPLC to detect the amount of cystine in our samples, a penicillamine-cysteine peak can also be detected, eluting just after cystine (refer to Figure 3.1). Further research into the ratio of these peak complexes may prove to be more useful, particularly in monitoring the effectiveness of patients on thiol-binding drugs.

Our genetic study revealed over 50 different mutations in our population, 23 of which have not previously been described. We did not find mutations in three patients suggesting there may be more genes involved in the disease or complex interactions between polymorphisms that we do not completely understand. More recently a new transporter in SLC7A13 has been described and performing DNA analysis looking for mutations in this gene may help us to understand the handful of patients without a mutation in SLC3A1/SLC7A9¹⁰⁰. More genome-wide studies are required to look for mutations in other genes. Whether or not a better understanding of these genes will allow us to develop gene or protein targeted therapies remains to be determined. Our

protein model may also allow a platform for which potentially new drugs can be tested.

Other groups have described using SLC3A1 and SLC7A9 knockout mice to facilitate the development and rigorous evaluation of novel therapies.^{29,122,123} The mouse model has been used in the testing of dimethyl ester (L-CDME), a cystine analogue that disrupts cystine crystal formation by attaching to their surfaces. CDME can reduce stone mass and size but has not yet been tested in clinical trials¹²⁴.

Further cystinuria research will require consensus on a validated method of measuring disease activity which can be facilitated by collaboration with groups such as the National Registry of Rare Kidney Disease (RaDaR)⁸⁴. Crucially, we need to define standards to aid in both research and clinical practice.

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